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## Political will now the issue

**THE** Commons Select Committee on Science and Technology has recently reported on the development of alternative sources of energy for the United Kingdom (House of Commons Paper 534). With painstaking care the committee has heard evidence on tidal power, solar energy, nuclear fusion, geothermal resources, wind and waves. And yet at the end of it all one is left wondering whether the United Kingdom is going to think itself into an energy policy and, if so, whether we will be prepared to bite the bullet on the subject of alternative energy sources.

The report itself is on the whole unobjectionable. Various sources are reviewed and commented on; solar energy for space and water heating is seen as having the greatest immediate potential, and the committee recommends its vigorous exploitation. Solar electricity is given a less cheerful assessment, although the rate of development of amorphous semiconductors might well render this opinion obsolete in the near future. Biomass, which is one of the pillars of Amory Lovins' *Soft Energy Paths* (offering according to Mr Lovins "practical economically interesting technologies sufficient to run an efficient US transport sector"), is barely mentioned. Wave power for electricity generation is seen, somewhat delphically, to have considerable potential "if the technical difficulties can be overcome, and economic viability established". Wind and geothermal energy are not highly rated. And so on. A disappointment is that there is no parallel assessment of energy storage techniques which, as Martin Ryle recently argued, could make a lot of difference to the viability of some alternative-energy projects.

Technical analyses of the sort that the committee provides are now common; there are plenty of excellent books, reports and magazine articles to inform the public, and this latest document can add relatively little to what has already been put about—and what fairly quickly becomes outdated. Where those in the political arena should now be more exercised is in asking just

what alternative energy sources are for. And before such a question can be answered some distinctions need to be made between different types of alternative energy source. To include nuclear fusion in the same general category as, say wind power is clearly wrong. The former has the potential at some time in the future to take over a massive proportion of the country's energy requirements if the political will is there, the latter does not, and it seems foolish to talk about such divergent technologies under the same heading.

Some clear thinking is also needed in the near future on what will be politically acceptable in terms of import and export. It has been customary to discuss some alternative-energy technologies as worth pursuing mainly for their export potential. But the opposite side of the coin is the possibility of importing energy. The question of biomass again springs to mind. Undoubtedly the United Kingdom is not favourably situated compared with equatorial countries. Many of these countries fall into the developing category. The policy questions of independence *versus* support for the developing world *versus* fear of a future OPEC are certainly worth pursuing at this early stage.

Finally, what is the purpose of research into alternative energy sources? If in future years the source could be expected to provide, say, 5% of the UK's energy needs, does it make sense to pursue the subject? Small indentations into a major problem may be attractive scientifically and emotionally but could be trivial in the context of resource conservation or reduction in expenditure on foreign currency. Somewhere along the line this far-from-easy problem will have to be faced; on the assumption that the United Kingdom will continue to place its energy reliance on the major sources in view at the present, for how long will it make sense to encourage vigorous research in the small alternative sources? The question would, of course, be very different if we were to move to a 'softer' path, but on that there seems as yet to be no political discussion. □



# BA address: Huxley responds

*Sir Andrew Huxley, Royal Society Research Professor in the Department of Physiology at University College London, responds to Nature's editorial on the presidential address he delivered to the recent British Association meeting*

SIR,—Your editorial (8 September) about my presidential address to the British Association misses the point of what I said on the relations between human values and scientific research. I discussed this matter in several contexts—evolution; paranormal phenomena; the Lysenko controversy; and the inheritance of human ability. You mention only the last, and you write as though I had simply called for more research in this field. You have misread me. What I was urging was that the conclusions of whatever research is done should not be biased by preconceptions or by external pressures. In recent years, this issue has been bedevilled by strong pressures, which in Europe and in most of the USA have come almost exclusively from those who insist that the genetic component is negligibly small. These pressures have taken forms which I hope you agree are improper—the baseless insinuation that there is necessarily something 'racist' in admitting the possibility of a genetic component; denial of freedom of speech; and physical attacks. It is easy to imagine similar pressures being exerted by others in the opposite direction; if this occurs I shall resist them just as forcibly, but as far as I know this has not been happening, at least in Europe, since the defeat of Hitler.

I did not say much about the good that might come from an increase of knowledge on the causes of differences in ability, partly because this was not the subject of my address, partly because I am no expert in these matters but also because I did not wish to buy off opposition to my main thesis by playing down the risk of uncomfortable passages ahead if, in any field, we allow evidence to be collected and published. In my view the independence of Science is something that should be accepted irrespective of the consequences that may appear to us to be likely. Human affairs are so unpredictable that it is more probable that good will be done by letting our beliefs follow from the evidence, than by deciding without the evidence what beliefs will lead to the best actions. Since you challenge me, however, I will set out some of the ways in which increased knowledge could do good.

You say "Questions are bound to be asked about motives for doing more research, especially as there is little doubt that inheritance does play at least some and maybe a very significant part in the acquisition of ability". One answer—already given in my address—is as follows. If inheritance plays no significant part, long-term policy need only aim to provide equal opportunity and to eliminate prejudice based on class and race. But if its role is "very significant", we must also build into our system compensatory advantages for groups who would otherwise feel excluded from a fair share of well-rewarded and respected occupations: 'affirmative action' as in the USA would be a step in this direction but other measures might be needed as well—perhaps for example up-grading those jobs which have the lowest requirements in scholastic attainment. Such policies would naturally be resented by the groups whom they do not favour (witness the case of Allan Bakke now before the US Supreme Court) and probably ought not to be vigorously implemented unless it is clear that a substantial genetic

component exists.

A second way in which further investigation could be valuable is the following: if there are no appreciable genetic differences, the best way to establish the fact will be to carry out genetic investigations. An example is that Shockley's approaches to the National Academy of Sciences in the United States led indirectly to a review of the position being undertaken under the auspices of a committee of the US Social Science Research Council, and the resulting book (J. C. Loehlin, G. Lindzey & J. N. Spuhler, *Race differences in intelligence*, W. H. Freeman & Co., 1975) gives prominence to two interesting genetic investigations, one based on correlation of IQ with blood groups in persons of mixed ancestry, and the other on the performance of illegitimate offspring of Negro and White US servicemen with German women during the occupation of Germany after World War II. Neither of these gave any support for a simple genetic difference between Negro and White.

Yet another reason to wish for greater knowledge is that, as understanding improves, so emotional reactions will become less. Suppose, for example, that a gene were indentified which, like the sickle-cell gene, confers some resistance against a tropical disease but whose compensatory disadvantage in the homozygote is a moderate drop in scholastic ability instead of a haemolytic disease: I believe this would be regarded purely as a piece of misfortune to those affected and in no way as a matter for shame.

It would be idle to suggest more specifically the way in which improved knowledge and understanding might be valuable. The chief characteristic of research is that its outcome is not known in advance. A large part of my address was devoted to examples where the progress of science was held up by a false assumption that a result could be deduced by extrapolation from what was already known. This part of my address you dismiss as "a lengthy aside", but it is highly relevant to the question of the causation of differences of ability. Our understanding in this field is still in a primitive state, and I would guess that there will be several unforeseen changes of view on both the environmental and the genetic side before an agreed position is reached. Meanwhile, what is needed is, on the one hand, for policy-makers to be conscious of this uncertainty, to be guided largely by humane considerations and by common sense rather than by predictions based on particular theories, and to avoid decisions liable to be harmful if unsuspected factors turn out to be at work. On the other hand, research should continue, with as little bias as can be attained and without premature claims on either side to have reached a final solution.

I retain a conviction—difficult no doubt to justify rigorously in each individual case—that an improvement in our understanding of a problem will in the long run improve our attempts to solve it. I find it deeply depressing that you, the Editor of the leading general scientific journal of the whole world, should in your final sentences tell us in effect that, in any field which is important enough to generate emotion, we ought not to undertake research until we are sure that its conclusion will be to our liking.

ANDREW HUXLEY, FRS

*The full text of Sir Andrew Huxley's address appeared in The Times Higher Education Supplement of 2 September*



# Desertification: another mirage?

Robin Sharp reports on the UN Conference which ended in Nairobi earlier this month

**D**ESERT rash is an ugly dermatitis which spreads in blotches and peels off bits of the world's skin. Having diagnosed it, traced it to no fewer than 45 sources and called it desertification, the United Nations has this month successfully patented a selection of medicines to control and cure the disease.

The UN Conference on Desertification (UNCOD) was very much a synthetic event, in the best sense of the term. Neither a scientific nor essentially a political gathering, it sought to bring together a range of disciplines and socio-political interests to create a new holistic approach to the problems of desert rash in the world's dry lands. The conference did not pretend to be inventing a new branch of science but rather a new conceptual framework for research and policy-making. Judging by the high degree of consensus among the 90 countries present on the ailment and its cure, this seems to have been achieved.

One danger with a newly-identified problem is that people too readily assume it needs new-fangled solutions. In the case of desertification, the teams of experts who produced the basic conference documents were cautious enough to recognise modern technology as one of the hazards as well as the hopes. Because of their fragility, the principal document noted, dryland ecosystems are particularly vulnerable to misapplied technology; and in the developing countries where acceptance by the local community is a critical factor, modifications of existing technology and practice are more likely to be effective than radical innovation.

In their official conference paper, 'Ecological change and desertification', Andrew Warren and Judith Maizels of University College, London, also warn that stories of technological success in dryland agriculture "should not be accepted complaisantly". Fifteen million hectares of land have been irrigated along the Indus River in Pakistan, for example, but an estimated two-thirds of this vast area is now affected by consequent waterlogging and salinisation.

The conference was presented with three other 'component reviews' covering technology, climate and human and social factors. All four were then summarised in an overview report, which

provided the basis for the first part of the conference debate. In fact, apart from various nuances and some disagreement over whether and how to define the neologism coined for the conference title, there was little dispute over the secretariat's analysis of the causes and effects of desertification.

## Bone of contention

The only real bone of contention was whether or not, as averred by Dr Mostafa Tolba, the conference Secretary-General, "man now has in his possession both the wealth of knowledge and adequate technical means to bring desertification to a halt and, in many instances, to reverse the process". The overview paper emphasised the same point, asserting that the knowledge and experience to tackle most aspects of the problem was "available right now".

This, however, was not good enough for the six national and regional science associations which held a seminar in Nairobi immediately before the conference to feed in their views. In their opinion it reflected an implicit overconfidence in the adequacy of existing technology, not to mention an underestimation of the human and economic costs which in the short-term could well rival the costs of the problem itself.

Both concerns drew attention in the debate, a number of countries pointing out that more basic and applied research was still needed in areas such as water harvesting, rainfall prediction, water desalination, alternative energy sources and water-crop relationships. Several delegates stressed the need for intensified efforts in the field of climatology and a closer working relationship between climatologists and agriculturalists at various levels.

One experiment which may eventually enable climatological events to be predicted in the world's dry regions is to be launched next year by the World Meteorological Organisation (WMO). Under its Global Atmospheric Research Programme (GARP), run jointly with the International Council of Scientific Unions, the WMO will make detailed worldwide observations of the atmosphere over a 12-month period. It is described as the largest scientific experiment ever undertaken internationally for peaceful purposes, using satellites, aircraft, ships, balloons and Press handouts, so they'd better come up with something.

## Plan of action

After agreeing that desertification has many causes and that most are man-made, the conference then moved on to consider what could be done about them. Though most of it may be no more than a set of guidelines for any government caring to take note, the UN conference formula these days makes it *de rigueur* to produce a 'plan of action'. And as these things go, the desertification action plan went pretty well. It recommended careful assessment and monitoring of the extent of desertification, proper management of water resources, rangelands, livestock and wildlife, urgent measures to overcome the loss of water and land caused by irrigation, special steps to improve soil conservation, national land-use planning, and—just to make sure that nothing was left out—"national systems for monitoring the human condition".

Focusing on the developing countries, the 'plan of action' called for measures to strengthen indigenous capabilities in science and technology, paying special attention to the rational use of dryland resources. This might include setting up or strengthening national scientific institutions concerned with desertification. The plan further urged that research be vigorously pursued on cheap alternative energy sources which could help to halt the alarming rate of deforestation caused by the pressure of demand for firewood in many dry countries.

Pilot project experiments could be set up to test the use of wind energy for generators and water pumps, and solar energy for water heaters and distillers, cookers, food dryers and refrigerators. On all these points the conference anticipated further action at the second UN Conference on Science and Technology to be held in two years' time.

The need for public awareness campaigns to educate people in general and dryland populations in particular about the sustainable use of their environment was emphasised by a number of countries. As the Australian delegation put it, the people contributing to the problem must be made aware of its existence and causes. On another tack, the Australians were scathing about the special desertification map produced for the conference. Not only did it contain serious errors, but at a scale of 1:25,000,000 it wasn't going to be much help and in any case the validity of mapping a set of dynamic processes was questionable.

## Less than enthusiastic

For similar reasons, they were less than enthusiastic about the use of information from remote-sensing satellites as an anti-desertification tech-



nique. Out of six transnational feasibility studies drawn up for the conference, two were devoted to the monitoring of desertification in south-west Asia and South America by Landsat imagery, using the Earth Resources Technology Satellite (ERTS). The use of satellite imagery thus came in for quite a bit of notice, but the Australians and others thought it had aroused "excessive expectations" and reckoned that for most purposes 'ground truth' observations were a much better bet.

The transnational feasibility studies were commissioned as the basis for possible inter-governmental agreements on large-scale regional projects to combat desertification. Apart from the two remote-sensing schemes, the studies provide for the planting of 'green belts' north and south of the Sahara, the management of livestock and rangelands in seven countries of the Sudan-Sahelian region, and management of the major regional aquifers in north-east Africa and the Arabian Peninsula. Though challenged on grounds both of cost and effectiveness, the Secretariat was able to claim that several governments had already signified their readiness to go ahead.

Inevitably in the volume of research amassed for this meeting there were a number of inconsistencies, plus a few worrying omissions. At some points in the documents and the debate, the need of developing countries for simple, low-cost technologies was carefully stressed, but elsewhere sophisticated remedies were advanced without much evidence whether those to whom they were addressed could or should afford them. Likewise, it was acknowledged that desirable changes in traditional farming and other forms of land use were subject to important economic, social and cultural restraints. Yet when it came to calculating how readily the threatened drylands would respond to the conference's action package, the implications of this seem to have been overlooked in favour of more measurable financial, scientific and administrative criteria.

### Two omissions

Two significant factors in the desertification process were glossed over in Nairobi, intentionally or otherwise. Firstly, though the UN Food and Agriculture Organisation (FAO) has a conference on land reform scheduled for next year, the degree to which feudal or other land tenure systems still contribute to desertification in several countries—for example erosion caused by peasants forced out of the valleys to farm marginal slopes—was studiously ignored. And again, no one asked aloud whether those governments determined to get their nomads settled

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### *Marginal living on the Saharan fringe*

down had analysed the long-term consequences of demobilising the people best equipped to balance the economic potential and the limitations of life on the desert margin.

Perhaps the secretariat was a bit too anxious not to ruffle any political feathers. In any event, for better or worse, this was one of the least politicised UN meetings in recent times. Apart from an Arab move obliging the conference to pretend that Israel's impressive record of desert reclamation did not exist, the proceedings were remarkably free of ideological entanglements.

Of course there had to be a bit of a crunch at the end, and, not surprisingly, it centred on cash and administrative machinery for the post-conference follow-through. True to form, the industrialised countries came out against the creation of any new international body for desertification which they might have to pay for. They were having nothing to do with the notion of an international tax to help finance the 'plan of action', and Dr Tolba knew the odds when he conceded at the start that there might be "some problems" in attempting to set up another Special Fund for the purpose.

In the end, as everyone expected, the follow-through responsibility was handed over from Dr Tolba, Secretary-General of the conference, to Dr Tolba, Executive Director of the UN Environment Programme. For whatever apprehensions there might be in various capitals about UNEP, it was the cheapest, neatest, most logical answer—and Dr Tolba was generally acknowledged to have done a masterly job in getting the show on the road.

So from its barrack-like HQ on the outskirts of Nairobi, enlivened with

outdoor murals of wallowing hippos and the like, UNEP will now have the task of pulling all the other UN agencies together to work out uniform methodologies, to coordinate and expand their research efforts on desertification and to build a solid organisational structure inside the conference's conceptual scaffolding. A mammoth task, one might say, but there's no doubt much has been accomplished in the two years leading up to the conference.

### Not too disappointing

By the time UNDP, UNIDO, FAO, UNESCO, WMO, WHO, the World Bank and others have reshuffled their budget headings to make room for this new arrival, even the present level of spending to combat desertification will probably not look too disappointing, given the many competing development priorities. And it should be worth much more if and when all the bits are linked into a concerted programme. Added to this, according to one estimate, are 15,000 or more researchers and 300 institutions around the world already involved in arid-land research.

With resources on this scale available to be mustered in a global campaign, the prospect of halting and pushing back the deserts might appear no longer a mirage. But there's still one more snag the conference tended to forget. By the year 2000, when the 'plan of action' is due to be fully implemented, the population of the world's drylands, now around 600 million, will be up to 1,000 million or more, with all the concomitant pressures this will bring. Those charged with the problem may therefore find themselves having to run very hard just to stand still. And come to think of it, that is a bit like chasing a mirage, after all. □

## BRITAIN

# Discomfiting prognoses

*The UK Science Research Council (SRC) published its latest annual report last week. Chris Sherwell outlines some of the difficulties*

BRITAIN'S Science Research Council sees itself as the country's only supporter of 'big' science. Research in high energy physics, astronomy, space and geophysics, it feels, would collapse if it withdrew its funding. The SRC is not about to pull out of these fields. But the pressure over the past few years to cut back has now reached such a pitch that the SRC believes it may not be able to discharge its responsibilities adequately by the early 1980s. Much now rests on whether some sort of turn-round materialises in the next couple of years.

Last week's report\*, covering the twelve months to March this year, continues to promote the image of unrelieved gloom that has become familiar in recent years, and in some respects offers even more discomfiting prognoses. Some idea of the worrying trend appears in the accompanying table, showing how the distribution of SRC expenditure will change. But the report also spells out in cold terms what is called the "best disposition" for big science over the coming years.

Nuclear physics expenditure, for example, will run down more steeply, at 6½% a year instead of the 5% planned last year. The £43.5 million to be spent this year will fall to £33 million by 1981-82, of which fully £20 million will go on the CERN subscription. This level of support, says the SRC, is inadequate to continue the full exploitation of existing facilities, and will not permit the full use of the nuclear structure facility at Daresbury or allow British participation in any future international high energy facility.

The more important question for now, however, is whether this level is below the 'minimum viable level' of support which became common parlance by last year but which went largely unspecified. That it is lower than forecast is certain; but different views will abound on whether in the process the minimum level has actually been breached or simply redefined. The high energy physics community's argument for the former would presumably be dampened by a more efficacious use of the CERN facilities.

Astronomy and geophysics fare

even worse than nuclear physics. Expenditure of £28 million this year, which the report says is 14% down on last year, will fall further still to £23 million in 1981-82. Space expenditure outside the ESA subscription (which remains at £8 million in all years) will fall from something like double that subscription to two-thirds next year and one-half by 1981-82. The report says this means proper advantage cannot be taken of opportunities through ESA and the use of the Space Shuttle and Spacelab.

The corollary, however, is that the domestic programme suffers. Such are the consequences of tight money that this obviously threatens to raise questions about the value of organised international efforts in space research. The alternative would be *ad hoc* use of US facilities. No one is saying publicly that Britain is even close to this position. But the signs are that such a state of affairs could loom in the future.

One comparatively bright spot focuses on the special programmes in the areas which the SRC has selected for support because of their national importance. Three in particular—the Teaching Company Scheme, Marine Technology and Polymer Engineering—collect a 25% increase from £14.5 million in 1977-78 to about £18 million in 1981-82.

A reminder of the sort of problems that big science projects pose is contained in the report's review of the Science Board's work. The Board is experiencing them for the first time with the conversion of Nimrod, the proton synchrotron at Rutherford, to a spallation neutron source—one of the SRC's now famous attempts at cannibalising existing facilities to save money. The Board chairman, John Jinks, says control of year-by-year expenditure was simple and policy was quickly effective when research grants were its main activity; with central facilities, on the other hand, long-term financial commitments, quasi-permanent operations and established staffs mean that delays are the only

way to economise, and this can vitiate the usefulness of the research.

How far the SRC has gone along the immensely fruitful line of cannibalising existing facilities is not certain. Plainly, though, the need to do it depends on financial exigencies. International subscriptions, which form some 30% of the SRC's total expenditure, are fixed; with the international value of sterling beyond SRC influence, and expenditure subject to the Treasury's cash limits regime, the SRC lacks control over a sizeable fraction of its outgoings. Couple this with inadequate compensation for increased student grants and for subscriptions, mix in the effect of public expenditure cuts last year and those planned for coming years, and the SRC has a recipe for lower expectations. What makes the fare unsavoury is the higher number of qualified candidates for studentships for whom lack of support means lost opportunities for advanced training.

A pick-up in the British economy is one way the outlook might theoretically improve. Other more logical and practical ways, at least from the SRC's point of view, include a greater commitment to the country's research effort on the part of the government, and a greater commitment to the SRC rather than the other research councils on the part of the ABRC. If there is cause for hope on the latter two of these fronts it may indicate an incipient 'backlash' effect following the SRC's past acceptance of negative growth rates (now -1.7% until 1981-82). The SRC wants a constant budget to do a competent job, and to achieve that between now and 1981-82 it says it needs £25 million. But as yet this would demand a turn-round in policy as well as a turn-round for the economy.

● In a display of acute deadline consciousness, the appointment of Professor Geoffrey Allen as chairman of the Science Research Council has, after an unusually lengthy delay, been confirmed by Shirley Williams, the Secretary of State for Education and Science. The four-year appointment, which takes effect this coming weekend, first became known in March this year, and followed press reports that included names of certain individuals who had allegedly been approached for the job and turned it down. The usual need for clearances contributed to the delay, but administrative hold-ups and the publicity that surrounded the selection did not help.

Professor Allen takes over from Sir Sam Edwards, who takes on a part-time appointment as chairman of the Defence Science Advisory Committee.

## Changes in approximate distribution of SRC expenditure under present programme

|                          | %1976-77 | %1981-82 |
|--------------------------|----------|----------|
| Astronomy,               |          |          |
| Space and Radio          | 22       | 18       |
| Nuclear Physics          | 36       | 26       |
| Science                  | 24       | 30       |
| Engineering <sup>1</sup> | 13       | 19       |
| Central Programmes       | 1        | 3        |
| Administration           | 4        | 4        |

\*SRC Report of the Council for the year 1976-77 (HMSO, £2.50)

<sup>1</sup> including radio propagation research



## COMECON

● A team from the Warsaw Institute of Plasma Physics and Laser Fusion, headed by Professor Sylwester Kaliski, Poland's Minister for Education and Science, has achieved the generation of neutrons by thermonuclear fusion using a concentric explosion with an exceptionally high degree of symmetry. The result was announced at an international conference in Prague last week and received considerable eve-of-conference publicity on Polish radio and television. According to the broadcast, this is the first published result of neutrons from thermonuclear fusion being obtained by pure explosion. The broadcast spoke optimistically of "extending the process to a commercial scale", but the figures indicate the production of only  $3 \times 10^7$  neutrons from  $10^{-7}$  g deuterium, and it is estimated that a level of some  $10^{17}$  neutrons would be required for commercially viable thermonuclear power generation.

● It is reported from Poland that, in spite of the amnesty of last July, repressive measures are still being taken against supporters of the Workers' Defence Committee. The WDC was set up a year ago to defend the rights of the Radom and Ursus workers suffering reprisals for their part in protests against soaring food prices. During the next few months, a number of WDC members, including several scientists, were themselves subjected to repression, but support for the WDC grew. At the beginning of June, a group in Wrocław, mostly from higher academic institutions, sent a letter to the government criticising the press campaign against the WDC and urging that sanctions against WDC members be withdrawn.

A letter to the WDC now claims that shortly afterwards reprisals began against the signatories. Three lecturers, including Dr Eugeniusz Porada, a mathematician, lost their jobs, ostensibly for trivial administrative reasons. The letter was signed by Professor Stanisław Hartman of the Mathematics Institute of the Polish Academy of Sciences; he and Professor A. Duda have been relieved of their posts as consultants to the special mathematics courses for gifted young people, founded in 1973 in several university cities.

● Last month, the supervisors of the Comecon Departments of Inventions met in Ulan-Bator, Mongolia, to discuss the problems of patent protection within the Comecon bloc. Until the late 1950s, the Comecon countries somewhat neglected the protec-

tion of inventions and innovations; since then, the position has changed so much that now even minor factory modifications are granted a patent or its equivalent. However, a survey of Comecon patent law, published in Warsaw in 1970, revealed considerable discrepancies in practice between the various countries. Thus, in the



USSR and Bulgaria, an 'author's certificate' gives unlimited protection; in most Comecon countries, the patent protection is for 15 years, in East Germany 18 years and in Hungary 20 years. The invention must be used within two years of its being reported in Hungary, and within three years of the granting of the protection in Poland; in the USSR there is no obligation to use an economic patent at all. In 1971, the Conference of Supervisors of Departments of Inventions was established to promote cooperation in the field of patents and to eliminate such anomalies.

The twelfth meeting of the conference, which was opened recently by the Mongolian Deputy Premier, S. Luvsangombo, and chaired by the Deputy Chairman of the Mongolian State Committee for Science and Technology, presented a promising picture of work in hand. A progress report was presented on the co-ordination of draft agreements on mutual legal protection of instructions of origin and designation of origins of commodities. A model bilateral agreement on conducting patent investigations was approved. Special attention was paid to problems of "rationalising" the economy, including the work of "rationalisers" within the framework of international economic organisations and a model system for handing over rationalisation proposals as part of scientific and technical cooperation agreements.

● One important element in Comecon integration plans is computerisation of both production and planning. As far as the production of components is concerned, the computerisation plans seem to be going forward well. Bulgaria has recently built up with Soviet aid a flourishing electronics industry, and last year earned more than 480 million foreign-currency leva from exports.

When it comes to the introduction of computers into the Comecon economies, however, the picture is less rosy. In Czechoslovakia, for example, the predominant trend so far has been the construction of data-processing systems for individual factories. These facilities, however, have been grossly under-used, the factory computers often simply recording data which is not applied in subsequent management and planning decisions. Recent figures from the Czechoslovak Federal Statistical Office indicate that on occasion factories have not used as much as two-thirds of the available machine-time, but either sold it to other consumers, or else simply wasted it. Accordingly, greater emphasis is now being placed on the construction of combined data-processing centres.

● Anti-pollution measures have recently been commanding a great deal of attention throughout the Comecon media. Thus Bulgaria, which recently introduced a set of "Basic Guidelines for the Conservation of the Environment", is to spend 78 million leva in Sofia *okrug* (county) alone, mainly on water-treatment plants on the Iskar, Marissa and Topolnitsa rivers, and on equipment to remove toxic gases from the air. This is clearly a matter of some urgency—last year in the Sofia *okrug* 106 industrial, economic and commercial enterprises had to be closed down, 25 of them permanently, for violating public health requirements.

In Hungary, air pollution surrounding the Metallokema works at Nagytetyeny has been causing concern since the middle of April. Complaints from local residents indicate that household pets were dying and fruit, vegetables and window-sills were covered with a greyish dust. In addition, three small children had to be treated in hospital for lead poisoning: an investigation by the local health authorities showed that the lead content of the atmosphere was 30–40 times the permitted level, and on 30 June the factory was ordered to close. The order was ignored.

Vera Rich



## IN BRIEF

## JET: Costly hold-up?

Confirming the loss of members of his group to the United States, Dr Paul Rebut, the French scientist who heads the design team based at Culham working on the European fusion project JET, was quoted as saying last week that even if a decision on the site of the next stage of the project was taken now, it could take up to a year to re-form the team.

Dr Rebut's comments, in an interview with the *Oxford Mail*, followed the news that the EEC Council of Foreign Ministers had not even discussed the subject, let alone come to a decision, when it met last week. The subject did not appear on the agenda because meetings between representatives from

Britain and Germany, the countries with the strongest candidates for the siting of JET, had not taken place as planned since the last Council meeting in July. A high level Anglo-German meeting in Bonn set for 9-10 September was cancelled because of the kidnapping of Hans-Martin Schleyer. The ministers will meet informally on 8-9 October, and formally on 17-18 October.

## NERC report, appointment

Like the UK SRC, the Natural Environment Research Council (NERC) last week published its report for the year to March 1977 and acquired a new chairman. A feature of the report is a

6-page description of the contribution NERC research can make to solving problems of energy and the environment; in particular, on the controversial issue of disposal of high-level radioactive waste, the report says the funds needed are "many times greater than the very modest sums" with which NERC is undertaking current studies.

The report also shows that the main bulk of the £26.675 million spent on NERC's scientific programme went on studies of the earth (42.2%) and the sea (32.9%). Commissioned research totalled nearly £15 million.

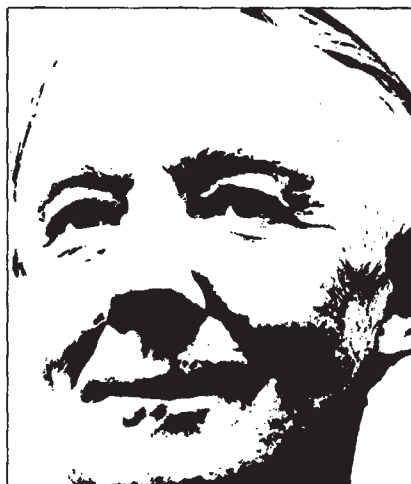
The man who will succeed Sir Peter Kent as chairman of NERC is Professor J. W. L. Beament, the insect physiologist.

MANY observers thought that the 1972 United Nations Conference on the Environment, in Stockholm, Sweden, was marred by the evident differences of opinion between the rich and poor nations on their priorities. The delegates from the so-called developed countries in Europe and North America were enthusiastic about controlling pollution (even when their governments were not always effective in carrying out these intentions). Many speakers from the poorer countries, now optimistically called 'developing', said that increasing their productivity and raising the standard of living of their citizens was more important. They pointed out that Britain and other comparatively wealthy countries had done little to control pollution during their industrial revolution, in the nineteenth century, and to impose unrealistic and expensive standards on those who were just setting out on the same road would hold them back unnecessarily.

It is encouraging to find that this view is not universally held. I am just back from Mauritius, where I was invited by its government to advise on environmental problems and their control. Mauritius, known to many only as the former home of the extinct dodo, and the place where the most valuable postage stamp was printed, is a volcanic island in the middle of the Indian Ocean. It covers some 760 square miles and has over 800,000 inhabitants. It was first a Dutch and then a French colony, until captured by the British in 1810. It remained a British colony until 1968, when it became an independent state, a member of the Commonwealth. Its main income is from sugar, and some 54% of the island's surface is covered by sugar cane. It

is a net food importer, and attempts to reach greater self-sufficiency by the diversification of agriculture are being made, though little good land remains uncultivated. Industrial development, particularly of light in-

## Dirty development



KENNETH MELLANBY

dustries, has recently made some progress, and is said to produce nearly a quarter of the island's income.

As yet Mauritius has no really damaging pollution problems. It would be hard to produce serious air pollution in a small oceanic island in the region of the trade winds. There is some smoke from factories, dust from stone crushers, and road safety is often impaired by the smoke screens from badly maintained diesel lorries and buses. The beaches of white coral sand, the main tourist attraction, are clean except near settlements and sewage outfalls. How-

ever, there are many at present trivial nuisances which could soon grow into environmental dangers. Refuse, likely to increase with population and a growing GNP, is seldom collected and never dealt with hygienically. Much is thrown into streams, the one place where the maximum damage will occur. Factories are surrounded by noisome piles of uncollected debris. Some people exhibit a genius for doing the most damage with the smallest amount of a pollutant. Thus a small tea factory in a sparsely inhabited hilly area has a leaky pipe from its oil tank. A few gallons seep out each year into the adjoining stream, half a mile above the intake for the local water supply. Garages always seem to empty their used sump oil into the nearest river.

Thus with a growing population, rising incomes and more industry the future for Mauritius could be a dirty one, unless immediate steps are taken to preserve the environment. Some encouragement can be obtained from the sugar industry, which could be a major polluter. The largest and best-run factories have remarkably little harmful impact on the environment, and the others are improving. The industry clearly knows what needs to be done. None of the other problems is insoluble—and success will be easier the sooner vigorous action is taken. In all developing countries prospects are better than they were some years ago. The most valuable aid that the developed countries can give is knowledge of how they have got over the abuses of the nineteenth century. They must show that with modern industrial development, pollution control need not be unduly expensive, and that early action can be an excellent investment.

# correspondence

## Europe versus itself

SIR,—With reference to the editorial "Million-dollar problem—billion dollar solution?" (14 July, page 89), the problem of sulphur pollution is not a problem of Norway versus the United Kingdom, but rather of Europe versus itself.

The OECD study, to which the article refers, has shown that there is a more or less continuous export and import of pollutants between European countries and that the problem is not confined to any two countries. Nor can it be solved by individual national control programmes. It should also be emphasised that the problem is not predominantly confined to the effects of acid rainfall on fish populations. The effects of sulphur pollution fall into four main areas: human health, vegetation, the fresh water ecology, and corrosion of metals, painted surfaces and other materials.

It is established that several categories of human disease are linked to the occurrence of sulphur dioxide and associated pollutants in the atmosphere. Governments' efforts to improve the quality of the air so as to provide better health protection are likely to have the reduction of sulphur emissions as one of their main targets.

With regard to corrosion we know that material damage due to sulphur pollution in Sweden and the United States cost between \$5 and \$10 per capita per year in the early 1970s. We know that damage is inflicted on vegetation in areas polluted with high concentrations of sulphur. It is also suspected that the dispersion of sulphur will result in decreased tree growth in areas with relatively low average concentrations of pollutant. The economic consequences of such effects can be highly significant.

We must also expect synergetic or combined effects in wide areas within Europe where various harmful pollutants are dispersed through the atmosphere. Efforts should now be directed at developing a coordinated European policy for reducing emissions. The costs involved will, it is true, be formidable. Only gradual improvement can therefore be expected. The result, however, will be beneficial to health, to materials susceptible to corrosion and to the quality of the natural environ-

ment all over Europe, with particular benefits accruing to people and areas closest to emission sources.

Yours faithfully,

ERIK LYKKE

Ministry of Environment,  
Norway

## Nutrition in food policies

SIR,—Blythe and Rush (4 August, page 386) outlined the state of the cautious dialectics of a UK food policy which incorporates nutritional criteria. The arguments are complex and controversial, not least because of the range of disciplinary interests involved. Three of the arguments referred to by the authors warrant further comment.

First, is the search for 'absolute proof' of causal linkages between dietary constituents and diseases necessary before recommendations commensurate with formulating food policy can be made? Is 'absolute proof' actually attainable? The scientific method sets out to falsify theories through experimentation, never to prove their validity, it is neither the intent of the method nor logically possible to prove the validity of theories, irrespective of the number of experiments. Thus, the argument should be concerned with the degree of confidence that can be attached to the corroboration between theories and observations in scientific experiments. Thus, for example, where there exists a sustainable conjuncture that a particular level of dietary fat (or saturated fat) is detrimental to health, this should be sufficient basis for a recommendation.

Second, the lack of an explicit food policy which links nutrition and health to food supply is tantamount to accepting, by default, that the fragmentary components of existing policies which affect food consumption adequately accommodate nutrition and health goals. Equally, the nutrition and medical professions, by failing to make recommendations, are by default condoning the present dietary trends.

Concerns that the introduction of nutritional goals into agriculture and food supply policy would require significant structural changes are exaggerated. The food supply industry is not static: at present there is a programme

for expansion of UK agriculture, and for further structural change within the EEC. There is no reason to believe that the changes needed to include nutritional goals would be any greater than those already under discussion or being implemented for sectional economic interests alone.

Thirdly, the controversy surrounding state intervention in consumers' freedom of choice through a food and nutrition policy is a red herring. The extent to which a real freedom of choice exists is academic, but it is evident that consumers' choice is directed by a wide range of measures from diverse sources. Effectively, consumers are free to choose from numerous, but pre-determined, alternatives, and that choice is further constrained by such factors as their income, access to information, and susceptibility to advertising. A food and nutrition policy would not necessarily impose further restrictions on choice, but could provide the consumer with a more rational and coherent basis for his choice. This implies that all consumers have access to a nutritionally adequate diet with established health safeguards, while maintaining the widest feasible variety and availability of foods. As a wide access to relevant information on nutrition, health and foods is a component of most conceptions of a food and nutrition policy, it can be argued that such a possibility would increase the consumer's capacity to exercise a free choice, rather than reduce it.

Yours faithfully,

C. J. ROBBINS

Reading,  
Berkshire

## Crater good

SIR,—I shall say an Ave Mare for the soul of David W. Hughes (15 September, page 197).

Yours faithfully,

L. ROSE

Leicestershire, UK

## Correction

In T. R. C. Boyde's quoted derivation of the word 'enzyme' (15 September, page 194), the Greek for 'in yeast' should have read *ενζυμη*, not *ενζυμηη*. The modern Greek would read *ενζυμοος*.



# news and views

## The ultraviolet sense in flies

from Jonathan Ashmore

MANY insects can detect light in the near ultraviolet. So also can humans but there are problems in explaining the much greater insect ultraviolet sensitivity in terms of what is known about the visual pigments. Rhodopsin is probably one of the most intensively studied membrane proteins, yet great gaps exist in the description of the chain of events starting with the photoisomerisation of the chromophore 11-*cis* retinal and ending with the signal appearing in the nervous system. But in both vertebrate and invertebrate systems the chromophore has always turned out to be retinal, the aldehyde of vitamin A. The peak absorption wavelength  $\lambda_{\max}$  for those rhodopsins which have been studied either in the membrane or in solution lies in the range 460 nm to 535 nm. Free retinal has a  $\lambda_{\max}$  of 378 nm, and its association with the protein by way of the usual protonated Schiff base would tend to move  $\lambda_{\max}$  to longer wavelengths only.

However, the insect ultraviolet peak measured in the photoreceptors of a number of species both by electrophysiological and microspectrophotometric methods occurs around 360 nm. A pigment from the moth *Ascalaphus* has been isolated by Hamdorf and co-workers (*Nature* **231**, 438; 1971) with a  $\lambda_{\max}$  at 350 nm and retinal as the presumed chromophore, but the matter is not settled there. In rhabdomeres R1-6 of the dark adapted dipteran ommatidium, spectral absorption peaks not only at 500 nm but also in the ultraviolet. The precise wavelengths are species dependent, but the ultraviolet absorption has peculiarities: the phenomenon appears to be fairly labile, varying from cell to cell and may even change seasonally (Horridge & Mimura *Proc. R. Soc. B* **190**, 211; 1975). Since its first description 15 years ago it is still not clear whether one or two pigments are present. The sharp angular sensitivity of the receptors seems to rule out the possibility

that there is coupling from the specifically ultraviolet receptors R7 and R8.

On page 386 of this week's issue of *Nature* Kirschfeld *et al.* sidestep the one/two pigment issue by suggesting that the ultraviolet sensitivity of dipteran photoreceptors may be due to a mechanism similar to that used to extend the spectral sensitivity of photographic emulsions. Performing the microspectrophotometry on rhabdomeres R1-6 of the housefly *Musca*, they extend the earlier work on *Calliphora* by Langer and Thorell (*Expl. Cell. Res.* **41**, 673; 1966) to show that although the ultraviolet peak occurs in absolute absorption spectrum there is no conspicuous change in the ultraviolet extinction following adaptation by ultraviolet light. Such a change is virtually a prerequisite for the presence of a rhodopsin-like pigment, and special mechanisms need to be invoked to explain its absence.

The first relatively stable reaction product of rhodopsin following the absorption of light is metarhodopsin. Insect metarhodopsins are distinguished by the large redshifts of the absorption peak relative to rhodopsin. In *Musca* the effect of absorption of ultraviolet light seems to be the production of only the metarhodopsin with  $\lambda_{\max}$  of 580 nm which is that formed from the blue-absorbing rhodopsin with a  $\lambda_{\max}$  of 490 nm.

Where is the ultraviolet light being absorbed? The new technique which has been used to study the mechanism further is electrophysiological. Pak and Lidington (*J. gen. Physiol.* **63**, 740; 1974) reported that the fast potential elicited by short intense flashes of light with external recording from the photoreceptors is proportional to the metarhodopsin concentration. This provides a direct assessment of the pigment state, whereas earlier work relied on the slower receptor potentials where several stages of transduction are involved before the electrical signal can be measured. Kirschfeld *et al.* find in *Drosophila* that those flies fed a vitamin A deficient diet have a lower ultraviolet quantum efficiency com-

pared to blue light than those flies raised on a vitamin A enriched medium. The method used can now leave little doubt that the vitamin A deficiency alters in some way the efficiency of ultraviolet light absorption at the level of the visual pigment itself. Species differences in visual performance between various dipteran species have been noted as a result of vitamin A deprivation (reviewed by Stark *et al. Naturwissenschaften* **63**, 513; 1976), but the differences between *Musca* and *Drosophila* are probably not significant here.

The novel explanation offered for these observations is that a photostable pigment is present in normal flies which absorbs in the ultraviolet and transfers energy to the chromophore on the rhodopsin molecule. Although vitamin A deficiency may be working by reducing the rhodopsin concentration (see for example Harris *et al. Nature* **266**, 648; 1977), the primary effect must be the reduction in retinal and its derivatives. A vitamin A derivative is thus a likely candidate for the photostable pigment postulated, presumably membrane-bound in view of the sensitivity of these receptors to the plane of polarisation of ultraviolet light (Horridge & Mimura *op. cit.*). Cattle rhodopsin has in fact been prepared artificially with two retinals bound and has a double absorption peak, one in the ultraviolet (Rotmans *et al. Biochim. biophys. Acta* **357**, 151; 1974). Functional studies of such a system have not been made but it is known that fluorescent probes can be attached to sites on the rhodopsin molecule (Wu & Stryer *Proc. natn. Acad. Sci. U.S.A.* **69**, 1104; 1972) whose behaviour suggests that the kind of energy transfer required to explain the insect data may be possible given a suitably close pair of sites.

Although not based on radically new data, the suggestion of a 'sensitising pigment' may be the solution to old problems. Further developments including a more direct attack on the protein chemistry involved should tell whether it is right. □



# Interferon action revisited

from Paula M. Pitha

INTERFERONS are species-specific cellular glycoproteins synthesised by a wide variety of animal cells in response to viral infection (or various non-viral stimuli) which induce antiviral resistance in homologous cells. In interferon-treated cells, a wide variety of different viruses do not replicate well. It was shown with a majority of the viruses studied that the primary effect of interferon is a general inhibition of viral protein synthesis. In addition to this block it has also been found that with some viruses such as VSV and SV40 the transcription of viral mRNA is decreased in interferon-treated cells. Oncornavirus replication seems to be inhibited by interferon at the level of virus maturation and assembly.

The nature of the molecular mechanism by which interferon induces the antiviral state in the cell has not yet been clarified. Interferon by itself is not an antiviral agent. The antiviral state does not develop in enucleated cells or in cells treated with actinomycin D or cycloheximide. This indicates that the antiviral effect of interferon requires both transcription and translation of the cellular genome and leads to the proposal that there is an interferon-induced antiviral protein (Taylor *Biochem. biophys. Res. Commun.* **14**, 447; 1964). The situation, however, is not that simple; recent results from the laboratories of I. Kerr (MRC, Mill Hill, London), P. Lengyel (Yale University, Connecticut) and M. Revel (Weizmann Institute of Science, Rehovot) with cell-free protein synthesising systems, indicate that interferon treatment induces the synthesis of several translational inhibitors.

The first step in the induction of the antiviral effect is the interaction of interferon with the cellular membrane of a sensitive cell, and it has been observed that the interferon binding involves both cellular gangliosides and glycoproteins (Besancon & Ankel *Nature* **252**, 478; 1974; Vengris *et al. Virology* **72**, 486; 1976; Kohn *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 3695; 1976). The cellular uptake of interferon is not required for the antiviral effect (Ankel *et al. Proc. natn. Acad. Sci. U.S.A.* **70**, 2360; 1973); the sensitivity to interferon seems to be determined by a receptor located at the plasma membrane. Using antiserum to interferon, which neutralised all free inter-

feron in the medium, Vengris *et al. (Virology* **64**, 410; 1975) demonstrated that for the development of the antiviral state interferon had to interact with the external cellular membrane of the cell producing interferon.

The results obtained by several groups (for example F. Ruddle at Yale University, Connecticut, and F. C. Chany at INSERM, Paris) using mouse-human cell hybrids demonstrated that the sensitivity to human interferon is governed by human chromosome 21. Trisomy 21 cells were markedly more sensitive to human interferon than normal diploid cells or monosomy 21 cells (Tan *et al. Science* **186**, 61; 1974; Chany *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3129; 1975). Antibodies to hybrid cells containing human chromosome 21 were found to inhibit the antiviral effect of human interferon in human cells. Revel *et al. (Nature* **260**, 139; 1976) suggested that chromosome 21 codes for the receptor for human interferon on the cell surface.

The species specificity of interferons seems to be also determined at the receptor level. Recent data indicate that in primate-mouse hybrid cells containing only one or a few human chromosomes interferon specificity is due to the interaction with the receptor but the antiviral mechanism induced with both mouse and human interferon is of mouse origin (Chany, *Biomedicine Express* **24**, 148; 1976). This indicates that at least some components of the antiviral effect do not have a requirement for species specificity. Samuel and Joklik (*Virology* **75**, 166; 1976) came to a similar conclusion when they showed that interferon treatment induced a ribosome-associated inhibitor of viral mRNA translation in cell-free systems that could be dissociated from ribosomes and inhibit the translation of viral mRNA in a heterologous cell-free protein synthesising system.

In this issue of *Nature* (page 422) Blalock and Baron from the University of Texas at Galveston present another type of evidence that the interferon-induced antiviral 'machinery' may not be species specific. The authors also suggest that the antiviral compound induced by interferon treatment of the homologous cells can be transferred to the cell of the heterologous species and elicit in it resistance to viral infection. The experiments are based on cocultivation of mouse cells (sensitive to mouse interferon) with interferon-insensitive human cells in the presence of mouse interferon, infection with

VSV or vaccinia virus, and measurement of virus yield. To achieve the effect close contact between the two cell populations was required; the effect was not observed in cultures plated at low cell density. Actinomycin D treatment of the mouse cells blocked both the antiviral effect in the mouse cells and their ability to transfer resistance to the heterologous cells. It seems, therefore, that the transferred compound may be a product of interferon induction rather than interferon itself.

This conclusion, however, is based on experiments done in mass cultures and the challenging viruses replicated both in homologous and heterologous cells. In the future much clearer data would be obtained if the infecting virus could not replicate in the cells homologous to the interferon but only in the cells to which the antiviral compound was transferred. It has been shown previously that the effect of interferon is an all-or-none phenomenon in L cells and in chick embryo cells (Chang *et al. J. gen. Virol.* **20**, 139; 1973; Radke *et al. J. Virol.* **13**, 623; 1974), and thus it should be possible to measure the transfer of viral resistance from the cell in the antiviral state to the adjacent neighbour. Fluorescent antibody or infectious centre techniques could be used to confirm the requirement for cell-cell contact.

One of the remarkable properties of the interferon system is its high biological activity. The specific activity of both human and mouse interferon is estimated to be close to  $10^9$  units per mg protein (one unit is the amount of interferon which inhibits virus replication by 50%). It is probable that such a system involves mechanisms which amplify the initial response. Both the above described transfer of antiviral resistance and the mobility of the interferon receptors on the cell surface (Lebon *et al. Proc. Soc. exp. Biol. Med.* **149**, 108; 1975) may be amplification factors in the interferon system.

Currently it seems that the action of interferon at the cellular surface resembles in many respects the action of the polypeptide hormones. The major task for the future is to answer the question of how the impulse from the cell membrane is transferred to the nucleus. One possibility which remains to be determined is that the antiviral effect of interferon is mediated through a second message such as cyclic AMP (Weber & Stewart *J. gen. Virol.* **28**, 363; 1975). □

# High-energy physics and nuclear structure

from C. J. Batty

A conference on high-energy physics and nuclear structure was held in Zurich on 29 August–2 September and organised by the Swiss Institute for Nuclear Research. This was the seventh in an international series of conferences held every two years. The theme of this conference series is the use of high-energy particles and accelerators to study the properties and interactions of nuclei and the use of low energy techniques and instrumentation to study the fundamental properties and interactions of elementary particles.

Of recent years this field has become increasingly dominated by the three 'pion factories' at LAMPF (Los Alamos), SIN (Zurich) and TRIUMF (Vancouver) and this conference was no exception, with all three accelerators producing a flood of papers on a very wide range of topics but with an

emphasis on proton and pion interactions with nuclei.

Of particular interest on the last day of the conference was a presentation of experiments from all three laboratories on the rare decays of muons. In an introductory talk L. Wolfenstein (Carnegie-Mellon University) briefly reviewed the gauge theories of the weak and electromagnetic interaction which have evolved from the work of S. Weinberg (who also spoke earlier in the meeting on this topic) and A. Salam. Wolfenstein pointed out that recent suggestions of the existence of a third lepton could lead to a coupling between the muon and electron such that decays of the type  $\mu \rightarrow e + \gamma$  could occur, although at a very low rate compared with the normal decay  $\mu \rightarrow e + \nu_e + \bar{\nu}_\mu$ . The question now is, do these rare decays occur?

All three laboratories presented status reports on experiments to measure the relative rate of the decay  $\mu \rightarrow e + \gamma$ . The work is technically very difficult and much of the discussion was concerned with presenting the many checks and detailed arrangements required to reduce the background to satisfactory levels and to ensure that any events seen which satisfied the

many selection criteria were true rare decays. The TRIUMF team have obtained an upper limit of  $3.6 \times 10^{-9}$  at 90% confidence for the relative rate. In a similar experiment at SIN in which  $6.3 \times 10^{11}$  muons were observed the upper limit obtained was  $1.6 \times 10^{-9}$  again with 90% confidence. In an attempt to reduce these levels even further a group at LAMPF led by H. L. Anderson are using a very high intensity muon beam with a magnetic spectrometer to detect the decay electrons. Running on the experiment has now started and the results are eagerly awaited.

In a related experiment a group at SIN have been looking for interactions of the type  $\mu^- + S \rightarrow e^+ + X$  using a sulphur target and a streamer chamber to detect the electrons. Again no true events have been seen and with the very low backgrounds achieved the group are able to set limits of  $4 \times 10^{-10}$  and  $1 \times 10^{-9}$  for reactions leading to electrons or positrons respectively.

However it would be wrong to give the impression that work from the three pion factories entirely dominated the conference. Measurements at Saclay of the scattering of 600 MeV electrons by  $^{208}\text{Pb}$  with the cross sec-

It is difficult to test experimentally the effectiveness of r and K selective pressures in the environment. Most of the evidence which has accrued in support of this useful concept has been circumstantial. For example, plant ecotypes of disturbed habitats often produce more seeds per plant and have a lower mean seed weight than their equivalents in stable situations (see *Nature* **262**, 351; 1976) and it is reasonable that one should account for this by reference to the selective pressures imposed upon populations. In a situation where disturbance, or calamity is frequent, the individual with a capacity for rapid and abundant seeding is more likely to have its genes represented in subsequent generations.

One of the clearest demonstrations of this in plants has been the work of Solbrig and Simpson on dandelions (*J. Ecol.* **62**, 473; 1974). In the triploid, apomictic species *Taraxacum officinale*, it has been possible to identify biotypes cytologically which are associated with different grassland conditions. Biotype A is found to predominate in disturbed sites and produce a larger number of smaller seeds than biotype D, which is more common in stable, undisturbed grass-

land. Experimental work has shown that although the two biotypes have similar growth rates in pure cultures, when grown together biotype D is more productive and has a lower mortality (see *Nature* **252**, 191; 1974). In other words, biotype D is more competitive in undisturbed experimental conditions.

Solbrig and Simpson have now (*J. Ecol.* **65**, 427; 1977) developed and extended this experimental approach to the investigation of r and K selection by introducing a disturbance factor

## Disturbed environments

from Peter D. Moore

into their experimental conditions. Seven plots of 2 m by 2 m were established and each was divided into four. Of the four subplots, two were planted with a mixture of A and D biotypes of dandelion and the other two with pure populations of each of the biotypes (four plants in each subplot). Screens were erected to prevent seeding from one subplot to another. In two of the seven plots the entire above-ground vegetation was removed (from all four subplots) in the middle of the second and the third growing season. In two more

plots, the above and below ground biomass was removed after tilling the soil (leaving only the residual seed in the soil). The remaining three plots were left undisturbed until the completion of the experiment at the end of the fifth growing season. All dandelions were then dug up, typed cytologically and weighed.

The results after final harvest showed that biotype D dominated the undisturbed plots; 85% of the plants were D type and constituted 91% of the biomass. In the plots where aerial biomass had been removed on two occasions, the situation was reversed and type A was dominant (93% of the plants occupying 93% of the biomass). A similar predominance of type A was found in the plots where all dandelion material (excepting seeds) had been removed. Type A was responsible for 92% of the plants and 97% of the biomass.

It seems therefore that the high competitive ability shown by biotype D in previous experiments indeed depends on no disturbance during the experiment. The balance between the two biotypes in a grassland habitat is evidently controlled by the degree of disturbance experienced. These experimental data therefore fully support the theoretical framework which is currently being assembled around the concept of r and K selection.

Peter D. Moore is a Lecturer in the Department of Plant Sciences, King's College, London.



tions spanning 11 decades have given charge distributions, and hence proton density distributions which significantly disagree with some of the best theoretical nuclear structure calculations. Measurements of electron scattering at high momentum transfer for non-zero spin nuclei give information on magnetic scattering which arises essentially from a single nucleon. Recent measurements of the radii of the orbits of these nucleons are again found to be in significant disagreement with theoretical calculations. It seems to be once again back to the computers for the nuclear structure theorists.

Studies of exotic atoms in which an electron is replaced by a heavier particle such as a muon, pion, kaon, sigma hyperon or antiproton are in progress at several laboratories. A collaboration of groups from the Universities of Birmingham and Surrey and the Rutherford Laboratory using the kaon beam from the Nimrod accelerator (now unhappily due to be closed down next June) reported the first measurements of the effects of the strong nuclear interaction on the line-shapes and energies of X rays from sigma-hyperonic atoms. Much of the discussion in this field however was focused on attempts to observe X rays from  $\bar{p}$ -p atoms where the usual electron in the hydrogen atom has been replaced by an antiproton. Teams working at CERN and Brookhaven have so far failed to see any evidence for X rays corresponding to 2p-1S atomic transitions. However a second team composed of physicists from CERN, Daresbury, TRIUMF and the University of Mainz also working at CERN claimed preliminary evidence for the observation of X rays leading to the 2p state. Theoretical calculations also presented at the meeting now suggest that the nuclear interaction in the 2p state may be such as to prevent the observation of 2p-1S transitions.

Although the evidence for X rays from the  $\bar{p}$ -p system is still controversial one of the groups at CERN presented some very direct evidence for the observation of high energy  $\gamma$  rays from quasi-stationary bound states of the  $\bar{p}$ -p system below threshold. Three narrow  $\gamma$ -ray lines were observed using a very large NaI crystal for detection. The existence of states of this type was predicted several years ago by a group of Russian physicists and their discovery, together with that of narrow resonance in  $\bar{p}$ -p interactions at energies just above threshold will give an added impetus to attempts at both CERN and at the Fermi National Accelerator Laboratory in the USA to

produce intense beams of low energy antiprotons for experiments of this type.

From these brief glimpses of just a few of the topics covered at the conference it can be seen that the discussions ranged over a wide variety of subjects. At a time when the two disciplines of high-energy (elementary particle) and nuclear (structure) physics seem to be growing even further apart the conference showed that the two topics have not entirely lost contact and that a very fruitful and productive liaison still exists. □

## The truth about the lower crust

from Peter J. Smith

WHAT is the physical and mineralogical structure of the lower continental crust? Several models have been proposed in the past, ranging from a fairly homogeneous layer of gabbro or basalt (based primarily on geophysical data) to a much more complex zone in which granulitic metamorphic rocks predominate (from geological evidence). Yet, local variations apart, both of these cannot be correct. Ultimately, all geochemical observations must be reconciled and integrated into a single consistent picture, which is what Smithson and Brown (*Earth planet. sci. Lett.* **35**, 134; 1977) have now attempted to do.

The 'simplest and most important conclusion' to be drawn from the new analysis is that the lower crust is certainly not homogeneous. Evidence for heterogeneity has been available for some time in the velocity variations and anomalies revealed by seismic refraction studies. But what really clinches the matter, according to Smithson and Brown, is the existence of numerous seismic reflectors in the lower crust. As they put it themselves: 'With no speculative interpretation at all, these reflections demonstrate the presence of numerous interfaces and a much more complicated lower crustal structure than has previously been supposed.'

Indeed, things are more complicated than even the mere presence of reflectors would suggest, for individual reflectors usually lack continuity. Moreover, the lower crust must on average be 'distinctly less mafic (and less dense) than gabbro', partly

because most seismic velocities found there correspond to granulitic facies rocks from granitic to intermediate composition and partly because if the most mafic rock type to be expected there in general is gabbro, more felsic rocks must also be present in abundance to provide the contrasts giving rise to the reflections.

What Smithson and Brown concluded from all this and more (for example, from direct observation of lower crustal sections now exposed) is that the lower crust can only be a complex series of metamorphic rocks interspersed with igneous bodies. Specifically, it probably comprises such rock types as granite gneiss, syenite gneiss, anorthosite, pyroxene granulite and amphibolite, all deformed, interlayered with both sharp and gradational contacts, and intruded by granite and gabbro. In such a predominantly metamorphic environment, isoclinal folding, abrupt changes in dip, distortion of layers, changes in layer thickness and igneous intrusion are only to be expected, offering an obvious explanation for the discontinuous reflectors observed.

The age of innocence is over as far as the lower continental crust is concerned. As a few people have consistently maintained, the geophysical simplicity has always been merely an illusion based on insufficient data. Not that geophysical data will ever be sufficient. For as Smithson and Brown point out, 'if the lower crust is metamorphic, not even high-resolution seismic reflection studies will ever reveal the detailed structure. Complex fold patterns... are unlikely to be resolved by any geophysical method.' □

## The nucleolus at Salamanca

from E. G. Jordan and U. E. Loening

The Fifth European Nucleolar Workshop of the European Cell Biology Organisation (ECBO) was held at the University of Salamanca on 27 June-1 July 1977, and was organised by Professor Giménez-Martín and others from the Consejo Superior de Investigaciones Científicas (CSIC), Madrid.

W. BERNHARD (Institute for Cancer Research, Villejuif) opened the workshop with the hope that the many specialities represented might be integrated, even if it was too early to speak of a synthesis, through a mutual respect and understanding between scientists.



The nucleolar rRNA genes provide an excellent system for studying transcription and gene organisation. The 'spreading technique' for visualising transcription in the electron microscope was first developed using nucleolar genes by Miller and Beatty. It has now been combined with autoradiography by N. Angelier *et al.* (Centre de Recherche, Ivry Seine) and S. Fakan *et al.* (Cancer Research Institute, Lausanne) to study transcription in nucleolar genes from amphibia and non-nucleolar genes from mammalian cells respectively. Autoradiographs of the typical nucleolar 'Christmas tree' transcriptional unit gave two surprising quantitative results. First, genes which were morphologically identical nevertheless showed different incorporation rates, indicating separate, single cistron control of the rate of transcription. Second, the latter part of the 'Christmas tree' covering the last third or so of the transcribed area showed no increase in the specific activity of the RNA. This may suggest that processing, the trimming of the transcript by nucleases, begins before termination.

One outstanding question is whether there are different sets of ribosomal genes. U. Scheer (Cancer Research Centre, Heidelberg) presented evidence for several size classes of transcription unit within the same organism although the apparent length of consecutive units on the same DNA axis seemed very uniform. M. Buongiorno-Nardelli (Embryology Institute, Rome) showed, in *Drosophila*, that the ribosomal genes of the same size class occur adjacent to each other in blocks and only selected size classes are amplified. Not only may the spacers and transcription units be different in length, but further confirmation that the rRNA precursor may be heterogeneous was again forthcoming. Scheer confirmed that the molecular weight of the pre-rRNA did not always agree with the transcript length and was sometimes heterogeneous. Supporting Scheer's electron micrographs D. Rungger and M. Crippa (University of Geneva) provided evidence that it is possible to detect RNA which hybridises to cloned ribosomal spacer DNA and that longer transcripts than the 40S pre-rRNA in *Xenopus* can be detected when processing is inhibited by fluorouridine. This suggests that transcription sometimes runs into the spacer.

A lively round-table discussion tried to put these and other results together; it is still not possible to define the primary transcript exactly since the pre-

rRNA may already be partially processed; no triphosphates have been found at the 5' start of the precursor; there may be some variability or multiplicity of initiation sites and read-through into the spacer, and perhaps a sliding or non-transcriptional movement or relocation of the polymerase carrying the RNP fibril. The mechanism and control of transcription of the ribosomal genes is clearly far from being understood despite the large amount of established data. The failure to find initiation *in vitro* with isolated nucleoli does not at present clarify anything.

However, as on every previous occasion, possible candidates for enzymes which process the precursor were described. M. Muramatsu (Tokyo University) had one; I. Grummt (Max-Planck-Institute, Munich) in collaboration with R. Crouch and S. Hall (National Institutes of Health, Bethesda) described a purified enzyme from the nucleolus which cleaves double-stranded RNA and converts the 45S precursor into distinct cleavage products; it is inactive under the conditions usually used for *in vitro* transcription by nucleoli. Perhaps this time a real processing enzyme has been identified. The spreading technique also provides evidence for the absence of nucleosomes in the nucleolar transcription complexes, which have the same length as measured on purified DNA (Scheer). This can be reconciled with the biochemical evidence for the presence of histones in the transcription complex by the idea that the nucleosome is a dynamic structure (reviewed by T. Tsanev (Bulgarian Academy of Sciences)) whose components may be rearranged in certain conditions. This would allow the conclusion that all the histones are present and arranged in units which can readily associate to form nucleosomes but which are sensitive to detergent and may only appear as a 3-nm fibril in spread preparations.

A clue to the way histones and nucleosomes might regulate transcription was presented by H. Busch (Baylor College of Medicine, Texas). By comparing the protein patterns from chromatin from active and inactive nucleoli he has discovered four proteins which markedly decreased in active chromatin. One of these proved to be a branched molecule containing a complete H2A sequence linked to the protein ubiquitin. One-fifth of the nuclear H2A could be accounted for in such molecules. He postulated that such a molecule in this proportion might easily have a key role in transcriptional regulation through the linking of nucleosomes in the stabilisation of the supercoils in condensed chromatin.

Various reports showed that both

HnRNA and nucleolar RNA synthesis is located at a cytological boundary. For HnRNA this is between dense and diffuse chromatin; for rRNA it is at the surface of the nucleolus organising region within the nucleolus.

Several speakers addressed the problem of how pre-messenger RNA is transported to the cytoplasm. It appears that it is wrapped in a wide variety of proteins, transported to the cytoplasm through the nuclear pore and there stored as protein-bound RNA or used immediately on polysomes. S. Penman (Massachusetts Institute of Technology) described a cytoskeleton (See *Cell* 10, 67; 1977) on which all cellular RNP is apparently translocated within the cell. Penman believes that this cytoskeleton provides a morphological explanation for the rapid interference with processing and translocation when transcription is inhibited and also accounts for the fact that RNA is never 'free' in the cell.

Support for the idea of such a cytoskeleton was provided by U. Lönn (Karolinska Institute, Stockholm) who reported microdissection experiments on the cytoplasm of polytene cells



## A hundred years ago

SOME of our readers may like to know that, as might have been expected, the three rhinoceroses now exhibited in the Alexandra Park are specimens of the African Black Rhinoceros (*Rhinoceros bicornis*). This species is extremely uncommon in menageries, and we have heard of no other in this country except the fine adult male now living in the Zoological Society's Gardens in Regent's Park.

It is perhaps a fortunate thing that our politicians, like the Chancellor of the Exchequer and Mr. John Bright, are beginning to concern themselves in their public addresses with science as well as art. With reference to Mr. Bright's recent address, as the *Times* remarks, if his hearers complain that they have not been told much about either science or art, we can only say that we agree with them, and that we deplore our common loss. In the coming time it is to be hoped that public speakers, like Mr. Bright, will know better what science really is than they seem to do now.

from *Nature*, 14, 27 September; 1876.

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which showed a gradient of ribosomes from nuclear envelope to plasma membrane, with the oldest ribosomes furthest from the nuclear membrane. The discovery of a very specific 28S rRNA nicking enzyme in erythrocyte cell membranes (M. Herzberg, Bar-Ilan University, Israel) fits with the idea that the lifetime of a ribosome is the time it takes to traverse this gradient.

The old idea of a link between the transport of messenger and ribosomal RNA which implies a role for the nucleolus in all RNA transport was supported by a report from S. Ghosh (Cancer Research Centre, Heidelberg) who showed that RNA transport was much lower in the absence of a nucleolus in artificially produced multinucleate cells.

It might be that the dogma that RNA polymerase A is confined to the nucleolus and polymerase B to the nucleoplasm does not hold any more. Penman questioned the former and a report from M. Laval (Cell Pathology Laboratory, Paris) using high resolution autoradiography, found evidence for polymerase B activity within the nucleolus. Such observations and a possible function in the transport of RNA make us cautious in limiting the role of the nucleolus to ribosome

manufacture.

Several speakers provided evidence that the so-called 'fibrillar centres' in animal cells and the lacunae of nucleoli in plant cells are in fact nucleolar organising regions. These reports precipitated a special session to discuss nucleolar nomenclature and agreement was reached that evidence for the presence of DNA in the fibrils of the pale staining regions, especially that from cytochemistry and autoradiography, and the presence of a synaptonemal complex at meiosis, justified abandoning the various vague and misleading terms 'fibrillar centre' 'pars amorpha' or 'lacunae' in favour of the nucleolar organising region. Bernhard observed that this represented an important advance since the last nucleolar workshop in 1975 in our progress towards an understanding of the nucleolus. It clarifies much of the earlier work and suggests a unifying concept for the interpretation of nucleolar ultrastructure.

Certainly this meeting will be remembered for the atmosphere of quiet scholarship in the midst of this beautiful old University city, and for considerable success in correlating structural with biochemical findings on the nucleolus and nucleus.

were reported in its diagnosis at an early stage. The test consists of the examination of oesophageal cell scrapings which are collected by a single-lumen tube or a double-lumen tube with an abrasive balloon (both of Chinese improved design). These on-the-spot techniques have made possible mass screening of patients, and the probability of detecting carcinoma in its early stages is now better than 90%. Its incidence in various areas of central and southern China has been correlated with possible carcinogenic factors such as local climatic conditions, hydrogeology, soil types and trace elements, diet and particular life-style. In some areas, which now serve as bases for experiments, the populace are voluntarily attempting to reduce their intake of nitrosamines and their precursors (suspected of contributing also to cancers of the bladder, kidney, liver, stomach and nasal sinuses), to minimise fungal contamination of food and to apply ammonium molybdate as fertiliser (nitrates and nitrites in food are suspected of being precursors to the nitrosamines), and the effects of such measures are being monitored. The principle 'put prevention first', it was emphasised, was paramount in the running of the Chinese health care system.

Another main topic of discussion was liver cancer. The method for its detection most widely used in China is the serum  $\alpha$ -foetoprotein assay, which requires only a drop of blood from the individual's ear or finger. Great success has been achieved in its early detection; specimens of it have been obtained which have not grown larger than 0.5 mm. In areas where it is common, efforts have been made to prevent mould contamination of food and improve drinking water, and to ensure early treatment of hepatitis by medical education. Other topics covered included the viral aetiology of nasopharyngeal carcinoma, to which southern Chinese people are particularly prone. Some medical workers reported that they have prepared several lymphoblastoid cell lines and found Epstein-Barr virus, and have successfully established an epithelioid cell line of the carcinoma.

Part of the conference was also devoted to cancer therapy and pharmacology. Over the past few years the Chinese claim many new anti-tumour drugs have been discovered. With the help of traditional medical knowledge some effective anti-cancer drugs, especially N-formylsarcosine and cephalotaxine ester, an alkaloid extracted from the plant *Cephalotaxis fortunei*, have been identified and their effectiveness is now being assessed in clinical practice. □

## Cancer conference in China

from T. B. Tang

A NATIONAL retrospective survey of cancer deaths is being compiled in China, on a scale which is probably unique hitherto. The investigation began in 1971 and was started first as a pilot project in Lian-axian in the north of Honan Province. That county is inhabited by some 110,000 people and has a high incidence of oesophageal cancer. The survey established that over a 30-year period the toll taken by the disease had been constant each year, at an annual average of between 1 and 1.5 per thousand. This is despite significant emigration and immigration into the district and improvement in living conditions since 1949, suggesting that the main contributory factors to this cancer are environmental.

The work has been subsequently expanded to cover the 50 million people living in Peking and three provinces of North China. Then, on the basis of experience from this extended survey, the incidence of a number of impor-

tant cancers was investigated nationally by the Academia Sinica. This programme involved eight of the Academia's research institutes and the 1.8 million barefoot doctors in the countryside. Up to now 500 million people in 16 provinces, municipalities or autonomous regions have been covered, and the research is still going on.

It is therefore interesting to note that, at the beginning of July, a national conference was held in Peking on the epidemiology of malignant tumours (*Hsinhua News Agency*, July 26; *Tai-kung-pao*, September 4, special report). Some 300 workers attended, including delegates from regional cancer research institutes, rural barefoot doctors, and practitioners of both traditional Chinese medicine and Western medicine. Experience was exchanged and summed up, these were put forward and debated on, and before the meeting concluded a work programme for 1977/80 was mapped out.

Carcinoma of the oesophagus remained one of the main concerns at the conference. Significant advances

# review article

## Recent developments in phase transitions and critical phenomena

David R. Nelson\*

*Universal dimensionless numbers characterise singularities at the critical point in magnets, binary mixtures and alloys, superfluids, and in a variety of other systems. A desire to understand these numbers has stimulated a significant breakthrough in the analysis of statistical mechanical partition sums. Critical phenomena can now be understood in terms of universality, Hamiltonian flows, and renormalisation group recursion relations.*

PHASE changes of matter in thermodynamic equilibrium are often accompanied by sharp discontinuities in quantities such as the magnetisation in a ferromagnet, the density in a liquid-vapour system, or the concentration in a binary mixture. Usually, these phase transitions can be made continuous by adjusting some external parameter such as the temperature. The point at which the discontinuity vanishes is called a critical point. At temperatures above the critical point, the spontaneous magnetisation of a ferromagnet is zero, the liquid and vapour phases of a fluid become indistinguishable, and the chemical species present in a binary fluid mixture are completely miscible. One usually associates the discontinuities below the critical temperature with some sort of broken symmetry<sup>1-3</sup>.

Although critical points were discovered in the nineteenth century, they have been the focus of particularly intense scientific investigation during the past few decades<sup>1-3</sup>. A rather precise characterisation of critical point singularities by experiments<sup>4</sup> and perturbation techniques such as high temperature series expansions<sup>5</sup> was followed by a detailed theory due to Wilson<sup>6</sup>. In this review, I shall describe Wilson's renormalisation group approach to critical phenomena, and illustrate its utility with examples chosen from the recent literature.

The spontaneous magnetisation and susceptibility of a ferromagnet are plotted schematically in Fig. 1. Below the Curie temperature, a substance such as iron or nickel will support a spontaneous magnetisation  $M_0(T)$ , which accounts for the well known magnetic properties of these substances. As the temperature is raised, however, the magnetisation drops to zero at  $T_c$  as shown in the figure. The magnetic susceptibility in zero field,  $\chi_0(T)$ , which is a measure of the response of the magnetisation to a small magnetic field, actually diverges to infinity as one approaches the Curie temperature from above or below.

The extraordinary thing about the anomalies shown in Fig. 1 is that they seem to be universal. That is, a wide variety of ferromagnets displays spontaneous magnetisations which tend to zero and susceptibilities which apparently diverge in precisely the same way. Despite differing chemical compositions, lattice structures, and Curie temperatures, one finds that  $M_0(T)$  behaves as

$$M_0(T) \sim |T - T_c|^\beta \quad (1)$$

near  $T_c$ , while  $\chi_0(T)$  diverges according to

$$\chi_0(T) \sim |T - T_c|^{-\gamma} \quad (2)$$

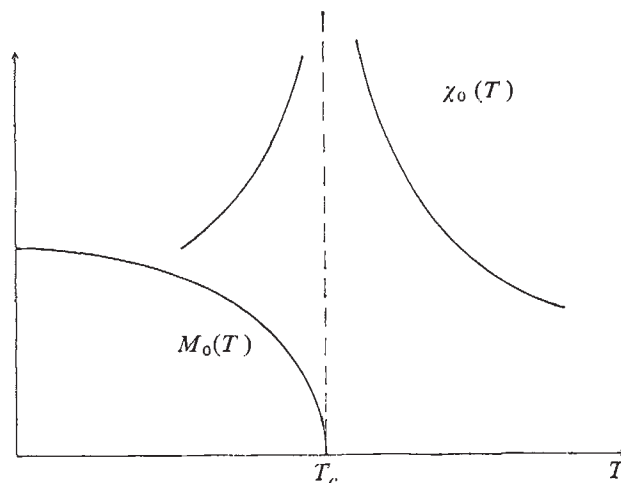
where the critical exponents  $\beta$  and  $\gamma$  are essentially the same for all magnets. A second curious fact is that the universal numbers  $\beta$  and  $\gamma$  are not simply one-half or unity, although most simple theories lead inevitably to these values<sup>1-3</sup>. In fact, experiments<sup>4</sup> and evidence from high-temperature series expansions<sup>5</sup> suggest that  $\beta$  and  $\gamma$  lie in the ranges,

$$0.31 \lesssim \beta \lesssim 0.38 \quad 1.25 \lesssim \gamma \lesssim 1.38 \quad (3)$$

in most substances. Although equation (3) indicates some variation in the observed values of  $\beta$  and  $\gamma$ , it now seems that magnets with the same symmetry should have precisely the same exponents: that is, if we imagine that a magnetic crystal is described by a lattice of classical spin vectors (see Fig. 2a), there is a weak dependence of critical exponents on the number of spin components  $n$  which actively participates in the phase transition. The number of such spin components is related to the symmetry group of the crystal.

The universality of critical phenomena is not limited to ferromagnetism. Analogous quantities to the magnetisation and

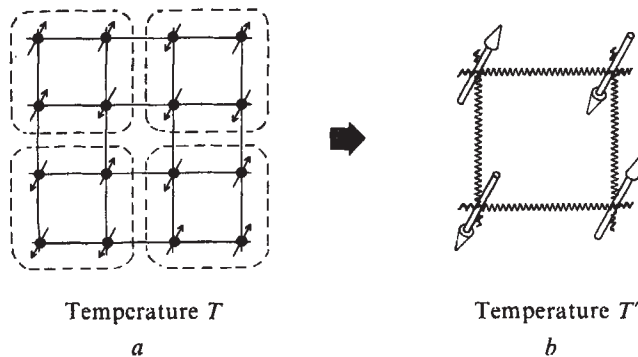
**Fig. 1** The magnetisation  $M_0(T)$  and zero field susceptibility  $\chi_0(T)$  of a ferromagnet. The magnetisation goes to zero at the Curie temperature  $T_c$ , while  $\chi_0(T)$  diverges as this temperature is approached from above or below.



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$$0.64 \lesssim \nu \lesssim 0.70 \quad (5)$$



**Fig. 2** Kadanoff block scheme. Dashed lines enclose groups of spins on lattice (a), which is at temperature  $T$ . Out of each block, we construct a collective spin variable by integrating over the internal degrees of freedom. The new spin degrees of freedom populate a lattice (b) with twice the old lattice spacing, and interact as if they were at temperature  $T'$ .

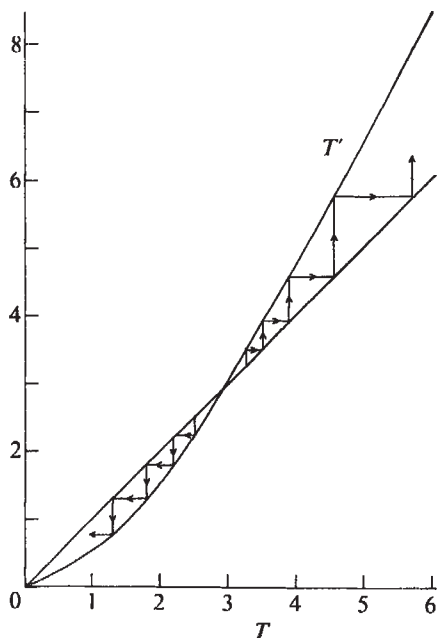
susceptibility exhibit singularities at the critical point of liquid–gas systems, binary mixtures, and alloys, at the  $\lambda$ -transition in superfluid helium, and at the Néel temperature in antiferromagnets<sup>1–3</sup>. Exponents such as  $\beta$  and  $\gamma$  which characterise the anomalous behaviour are very close to the numbers found for ferromagnets. A variety of other exponents can be defined for systems near a critical point, and they, too, seem to be universal.

The behaviour of a liquid–gas system near its critical point is particularly striking. The fluid suddenly becomes milky and opaque, and scatters laser light intensely in the forward direction. It seems clear that some sort of cooperative phenomenon is taking place, and that the particles of fluid are strongly correlated over distances comparable with the wavelength of the laser light. The correlation length  $\xi(T)$  of the fluid, which is a measure of the cooperativity, is expected to diverge strongly at  $T_c$ .

$$\xi(T) \sim |T - T_c|^{-\nu} \quad (4)$$

The exponent  $\nu$  is in the range

**Fig. 3** Approximate recursion relation for  $n = 1$  or Ising spins, with a fixed point at  $T^* = T_c = 2.98$ . The 'ladder' construction shows successive temperatures produced by repeated iterations of the transformation. The slope of the function  $T'(T)$  through its fixed point is related to the critical exponent  $\nu$ .



in most substances.

Where do the universal exponents  $\beta$ ,  $\gamma$ , and  $\nu$  come from? What is their precise numerical value? Can we account for slight variations, if any, in  $\beta$ ,  $\gamma$ , and  $\nu$  from system to system? A desire to answer these puzzling questions culminated in a major breakthrough in 1971. Wilson's renormalisation group theory of critical phenomena<sup>6</sup>. Building on work by Kadanoff<sup>7</sup>, Wilson showed why the exponents were universal and how to calculate them. In doing so, he placed at the disposal of condensed matter physicists a powerful mathematical tool, the renormalisation group.

### Kadanoff blocks and lattice renormalisations

If critical exponents are indeed universal, one might hope to understand them using only a very simple model of magnetism. Consider for concreteness a  $d$ -dimensional hypercubical lattice populated with fixed length, classical spin vectors  $S_i$  (see Fig. 2). To each configuration  $\{S_i\}$  of spins we assign an energy,

$$H[\{S_i\}] = -J \sum_{\langle i,j \rangle} S_i \cdot S_j \quad (6)$$

where  $i$  and  $j$  label the sites of the lattice, and the sum is restricted to nearest-neighbour pairs of spins. Each spin has  $n$  components, where  $n$  can be different from  $d$ , the dimensionality of space. According to the rules of statistical mechanics, the macroscopic properties of such a system follow from a calculation of the partition function,

$$Z = \text{Tr}_{\{S_i\}} \exp(-\beta H[\{S_i\}]) \quad (7a)$$

$$= \text{Tr}_{\{S_i\}} \exp\left(-\frac{1}{T} \sum_{\langle i,j \rangle} S_i \cdot S_j\right) \quad (7b)$$

In passing from (7a) to (7b), we have combined  $\beta$  (not to be confused with a critical exponent!) and the coupling constant  $J$  into a dimensionless 'temperature',  $T \equiv 1/\beta J$ . The trace operation ( $\text{Tr}$ ) means an integration over all spin configurations  $\{S_i\}$  consistent with the fixed length requirement, namely

$$|S_i| = 1, \text{ all } i \quad (8)$$

The partition sum (7) can be computed exactly in one dimension for all values of  $n$  (ref. 8) but does not exhibit a finite temperature phase transition. Onsager obtained the partition function in two dimensions<sup>9</sup> for the special case of the Ising model, or  $n = 1$ . Onsager's work, which remains today a true mathematical *tour de force*, eventually led to the exponent predictions<sup>1–3</sup>

$$\beta = \frac{1}{8}, \quad \gamma = \frac{7}{4}, \quad \nu = 1 \quad (9)$$

An analytical evaluation of (7) for other values of  $n$  and  $d$  seems to be a hopeless task.

Rather than attempting to evaluate (7) exactly, we can, in fact, make progress by merely thinning out the spin degrees of freedom slightly. To do this, we follow the original suggestion of Kadanoff<sup>7</sup> and group the spins on the original lattice (which is at temperature  $T$ ) into blocks as shown in Fig. 2. With some ingenuity, it turns out to be possible to integrate approximately over the internal degrees of freedom within each block. We are then left with a new statistical mechanical problem with twice the lattice spacing. Provided the interactions between spins in the new lattice are of the same form as in the old, couplings in the new lattice can be characterised simply by a new temperature  $T'$ .

This sort of program was first carried successfully for Ising ( $n = 1$ ) spins on a triangular lattice by Nicmeyer and van Leeuwen<sup>10</sup>. A very primitive version of their transformation gives a relationship between the new and old temperatures, namely<sup>10</sup>

$$T' = \frac{1}{2} T (e^{3/T} + 3e^{-1/T})^2 / (e^{3/T} + e^{-1/T})^2 \quad (10)$$

Equation (10) is depicted graphically in Fig. 3, together with a

'ladder' construction which shows how the temperature changes with repeated iterations of the transformation. The recursion formula (10) has a fixed point at  $T^* \approx 2.98$ , and it seems natural to identify this isolated point with the Curie temperature  $T_c$  of the spin system.

We can see the utility of this procedure of 'thinning out' spins as follows: even an approximate calculation of partition function (7) is difficult near  $T_c$  because of the large correlation length  $\xi(T)$  in this region. Note from Fig. 3, however, that the renormalisation transformation increase temperatures which, initially, are slightly above  $T_c$ . By repeatedly applying the transformation (10), we can relate a difficult calculation near  $T_c$  to a more tractable high temperature problem. A very accurate calculation of the partition sum (7) is possible at high temperatures by expanding in powers of  $1/T$ . No information about  $Z$  is lost during the thinning out procedure, because there is a very precise relationship between the partition function calculated at temperature  $T$ , and the corresponding quantity calculated at temperature  $T'$  (refs 6 and 10–12). In a similar fashion, we see that the transformation (10) decreases temperatures initially slightly below  $T_c$ , and eventually produces a more manageable (low temperature) problem.

As emphasised by Wilson<sup>14</sup>, the physics behind a renormalisation transformation is in the reduction of the correlation length. If the lattice spacing is changed by a factor  $b$  by the thinning out process, it follows the correlation length transforms according to<sup>14</sup>

$$\xi(T') = \xi(T)/b \quad (11)$$

For the square lattice spin system shown in Fig. 2,  $b = 2$ , while for the triangular lattice of Niemeyer and van Leeuwen (it turns out that)<sup>10</sup>  $b = \sqrt{3}$ . Equation (11) can be used in conjunction with (10) to produce a critical exponent. Near the fixed point  $T^* = T_c = 2.98$ , equation (10) can be written in the approximate form,

$$T' - T_c = b^{0.89} (T - T_c) \quad (12)$$

which when combined with (11) gives a functional equation for  $\xi(T - T_c)$ ,

$$\xi(T - T_c) = b \xi[b^{0.89} (T - T_c)] \quad (13)$$

It is easy to check that the solution of this functional equation is  $\xi(T) \sim |T - T_c|^{-\nu}$  with  $\nu \approx 1.12$ , which is not too different from the exact result  $\nu = 1.0$ .

In more sophisticated calculations<sup>10,15–18</sup>, it is not possible to describe a renormalisation transformation in terms of a single coupling constant. More complicated couplings such as next-nearest-neighbour and multispin interactions are generated after one iteration. When these extra couplings are taken into account<sup>10,15–18</sup>, they not only produce more accurate estimates of critical exponents, but also provide a qualitative explanation of universality: if several couplings are included in the calculation, the renormalisation transformation can be viewed as a mapping of one Hamiltonian on to another in a multidimensional coupling-constant space. As  $T$  is adjusted towards  $T_c$ , it turns out that any initial set of couplings 'flows' toward a unique fixed point under repeated applications of the transformation. Just as in the one-interaction-constant example, critical exponents are related to eigenvalues of the transformation about this fixed point. We are led to the conclusion that the exponents are independent of the precise values of the initial couplings, and depend instead on the 'universal' properties of the fixed point.

Calculations by the Kadanoff block method for Ising spins have now become quite refined. Niemeyer and Van Leeuwen<sup>10</sup> presented a series of cluster approximations to an exact transformation which seems to converge to the exact results of Onsager. Wilson<sup>15</sup> carried out renormalisations on a square lattice keeping track of 217 different interaction constants, and obtained critical exponents accurate to better than 1%. Kadanoff has proposed a variational principle<sup>17,18</sup> which, when coupled with a simple block

spin transformation, gives exponents accurate to 0.1% in two dimensions, and results competitive with high temperature series expansions in three dimensions!

Despite some initial success, this field is still in its infancy. The principal achievement of the block spin analysis so far (beyond providing an intuitive understanding of universality) has been to reproduce the results of Onsager and high temperature series expansions. Nevertheless, lattice renormalisation groups may eventually become a powerful computational tool, similar to various methods used to evaluate integrals numerically in ordinary calculus. The partition sum (7) can be viewed as a complicated functional integral which until now could only be computed in a few fortuitous cases. Renormalisation theory suggests that such sums can be evaluated routinely and very accurately at any temperature, without relying on a mathematical *tour de force* such as Onsager's solution<sup>9</sup> of the two-dimensional Ising model.

## Continuation in dimensionality

Wilson has characterised developments in the theory of critical phenomena as a "search for analyticity". The idea is to find a mathematical framework in which critical point singularities are described by smooth, analytic functions. Landau<sup>19</sup> proposed that critical phenomena could be understood in terms of an analytic expansion of the free energy in powers of a local magnetisation variable. Although Landau's theory has led to a qualitative understanding of a wide variety of critical points, the exponent predictions disagree with experiments<sup>1–3</sup>.

The proposal that critical points can be understood in terms of analytical recursion relations<sup>6</sup> seems to be the correct modification of Landau's theory. The method of construction ('thinning out' degrees in freedom) virtually guarantees that the recursion relations will be analytic at finite temperatures. Wegner<sup>19</sup> has shown that a wide variety of critical point singularities follow from the analyticity of recursion relations about a fixed point.

Perhaps the most useful analyticity property of recursion relations is that they are remarkably easy to continue in dimensionality. The original continuation of Wilson and Fisher into  $4-\epsilon$  dimensions (Landau's theory turns out to be correct above  $d = 4$ ) led to quantitatively accurate estimates of exponents in three dimensions, and to a detailed understanding of a wide variety of complicated critical point phenomena. The formulas for  $\beta$  and  $\gamma$  as a function of  $n$  and  $d$  ( $d < 4$ ) are<sup>21,22</sup>

$$\beta = \frac{1}{2} - \frac{3}{2(n+8)}(4-d) + \frac{(n+2)(2n+1)}{2(n+8)^3}(4-d)^2 + \dots \quad (14a)$$

$$\gamma = 1 + \frac{n+2}{2(n+8)}(4-d) + \frac{(n+2)(n^2+22n+52)}{4(n+8)^3}(4-d)^2 + \dots \quad (14b)$$

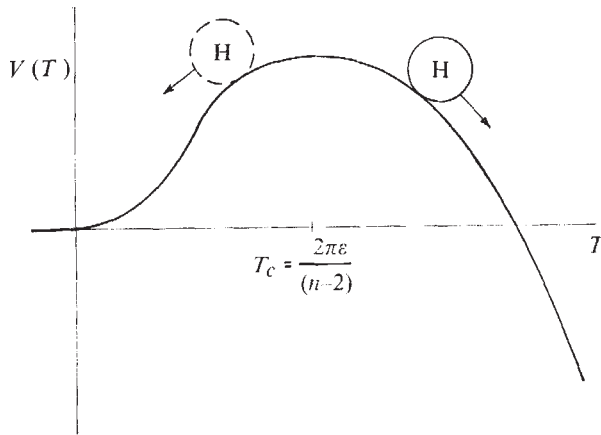
Although expansions like these in  $\epsilon = 4 - d$  (and, to a lesser degree, expansions in  $1/n$ )<sup>23–25</sup> probably represent the most powerful and versatile application of Wilson's theory, this work has been extensively reviewed elsewhere<sup>14,26–28</sup>. We shall turn instead to more recent investigations of critical points in  $2 + \epsilon$  dimensions.

## Critical phenomena in $(2 + \epsilon)$ dimensions

To a first approximation, the action of a renormalisation group on a Hamiltonian can be compared with the motion of a ball rolling down a hill<sup>6</sup>. One assumes that the differential change in temperature  $dT \equiv T' - T$  associated with a block spin transformation that changes the lattice spacing by a factor  $dl \equiv db/b$  is described by a potential function  $V(T)$  (ref. 6)

$$\frac{dT(l)}{dl} = -\frac{dV[T(l)]}{dT(l)} \quad (15)$$

Here,  $dl$  is an infinitesimal change in the logarithm of the Kadanoff block size, and  $T = T(l)$  is the effective temperature produced by



**Fig. 4** Potential function  $V(T)$  for fixed length spins in  $2 + \epsilon$  dimensions, with  $\epsilon$  small and  $n \geq 3$ . Hamiltonians initially to the right of  $T_c$  'roll' toward higher temperatures, while systems with temperatures less than  $T_c$  roll toward a zero temperature fixed point.

blocks of size  $b = c^l$ . Although a differential change in the block size is hard to visualise, it turns out to be fairly easy to effect this transformation mathematically.

Recent studies of the fixed length spin Hamiltonian (7) near two dimensions<sup>29-34</sup> provide a particularly simple realisation of these ideas. The potential  $V(T)$  has now been calculated by using a low-temperature spin-wave theory, with the result<sup>29,31</sup>

$$V(T) = \frac{1}{2}(d-2)T^2 - \frac{n-2}{6\pi}T^3 + \dots \quad (16)$$

As shown in Fig. 4, Hamiltonians at temperatures initially to the right of the crest in this potential roll toward high temperatures, while Hamiltonians at lower temperatures roll toward the minimum at  $T = 0$ . There is no oscillatory motion about this minimum because the 'equation of motion' (15) contains only one derivative in the time-like parameter,  $l$ .

This is precisely the behaviour we found using the discrete recursion formula (10), and it is natural to associate the crest of the potential hill with the critical temperature,

$$T_c = 2\pi(d-2)/(n-2) \quad (17)$$

In contrast to the  $n = 1$  or Ising model,  $T_c$  tends to zero as  $d$  approaches two from above for  $n \geq 3$ . Linearisation of the equation (15) about the maximum in the potential leads to the correlation length exponent prediction<sup>29-31</sup>

$$\nu = \frac{1}{(d-2)}[1 + O(d-2)] \quad (18)$$

Higher order corrections to this result are determined in ref. 31.

Note that for the interesting case of an XY model ( $n = 2$ ) in precisely two dimensions, the potential (16) is perfectly flat. This result (which holds to all orders in  $T$ ) suggests that such a system would exhibit a line of critical points with continuously variable critical exponents<sup>35-36</sup>. Work by Berezinskii<sup>37</sup> and by Kosterlitz and Thouless<sup>38,39</sup> gives convincing evidence that this line actually ends at some finite temperature. Renormalisation group flow patterns for the XY model obtained using a more sophisticated theory by Kosterlitz<sup>39</sup> and later by José *et al.*<sup>40</sup> are plotted in Fig. 5, which shows the motion of the temperature coupling  $T(l)$  together with the motion of a new parameter  $y(l)$ , which gives the probability of a vortex pair excitation in the system. These flows suggest that vortices become unimportant at low temperatures, so that spin-wave theory (which neglects vortices) should be qualitatively correct in region I. The instability in the flows in regions II and III, however, suggests a finite temperature transition into a phase not describable by spin-wave theory. It is possible that this bizarre phase transition may actually be observable in superfluid <sup>4</sup>He films.

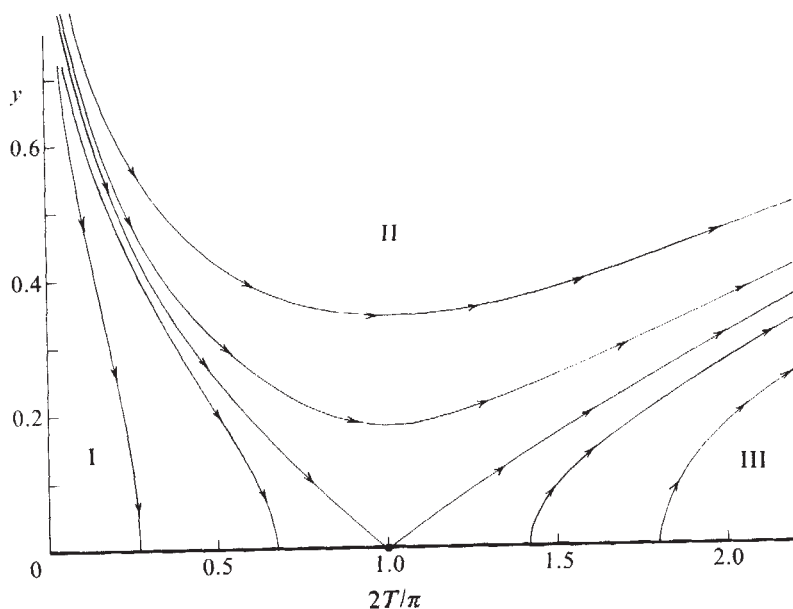
### Concluding remarks

We have seen that the renormalisation group is a rather powerful and sophisticated tool for understanding the behaviour of matter near a critical point. Much of the progress in this area resulted from a rather fertile interaction of statistical mechanics with quantum field theory, where the renormalisation group was first developed<sup>41</sup>. Renormalisation groups constructed for problems in statistical mechanics may eventually be of some use in understanding certain lattice quantum field theories<sup>42</sup>.

Modern renormalisation theory may also be useful in other areas of condensed matter physics. Anderson<sup>43</sup> and Wilson<sup>15</sup> have applied renormalisation group ideas to the Kondo problem, while Licciardello and Thouless<sup>44</sup> and Wegner<sup>45</sup> have used these techniques to study localisation in metals. Equilibrium time-dependent critical phenomena have been successfully treated<sup>46</sup> using Wilson's ideas, and there is now some effort to extend these techniques to non-equilibrium problems. One might hope that they would be of some utility in understanding, for example, properties of turbulent fluids at high Reynold's number.

Much of my understanding of static critical phenomena is the result of stimulating interactions with Michael Fisher, Kenneth Wilson, and Leo Kadanoff.

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**Fig. 5** Hamiltonian flows for the two-dimensional XY model. The flowlines show the motion of the coupling constants  $T$  and  $y$  under repeated iterations of a renormalisation group transformation. The parameter  $y$  measures the probability of exciting a vortex pair in the system. The flowlines are both attracted and repelled by a line of fixed points at  $y = 0$ . Two separating trajectories divide the plane of possible Hamiltonians into three distinct regions. Vortices are unimportant to the critical properties in region I, but become increasingly important in regions II and III.



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## articles

# The Messinian salinity crisis and evidence of late Miocene eustatic changes in the world ocean

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*The Mediterranean Sea became isolated from the world ocean in the late Miocene and underwent a 'crisis of salinity' during which vast deposits of evaporites were laid down in pre-existing depressions. The final connection with the Atlantic is believed now to have been the Betic Strait entering into the Mediterranean from Andalusia, Spain. Although the closing of this connection, known as the Iberian Portal, is related to large-scale plate movements which brought Africa into direct contact with southern Europe, its final severing may have resulted from glacial-eustatic lowering of the global ocean. Further stratigraphic resolution of late Miocene sea-level and ice-volume changes is sought to verify the eustatic-fall hypothesis.*

RECENT research, particularly that of specialists contributing reports on the DSDP legs 13 (refs 1, 2) and 42A (ref. 3) has revealed the existence of 1.5-2 km of evaporites throughout the deep Mediterranean basins and has strongly supported Ruggieri's<sup>4</sup> suggestion that a 'crisis of salinity' overwhelmed the faunas of the ancient Tethys Sea in latest Miocene (Messinian) time some 6.5-5 Myr ago (Fig. 1).

The salinity crisis occurred after the western Tethys lost its last connection with the Atlantic about 6.2 Myr BP (ref. 5), its connection with the Indo-Pacific having been severed at about the end of the Early Miocene<sup>6</sup> some 10 Myr earlier. The Iberian Portal, as the hypothetical last connection with the Atlantic is often known, must have existed somewhere between the Iberian Meseta and the Moroccan Meseta and could have been any one of the former Betic, Gibraltar, or Rif straits, although Benson<sup>7</sup> now believes that its connection to the Atlantic lay across Andalusia and was therefore the northernmost of the three passages. Isolation of the ancient Mediterranean from the world ocean resulted in the precipitation of more than 10<sup>6</sup> km<sup>3</sup> of gypsum, halite and other salts from a volume of seawater estimated to be equivalent to thirty times that contained in the present Mediterranean basins<sup>8</sup>.

The connection between the Atlantic and the Mediterranean may have been broken by plate movements bringing Africa into direct contact with southern Europe<sup>9</sup> or by uplift of the sea floor associated with the Alpine Orogeny (doubtless two aspects of the same basic process). It is possible, however, that the final separation of the Mediterranean from the Atlantic could have been brought about by a fall in the level of the world ocean<sup>10</sup> or by a combination of local uplift of the Iberian Portal and global eustatic

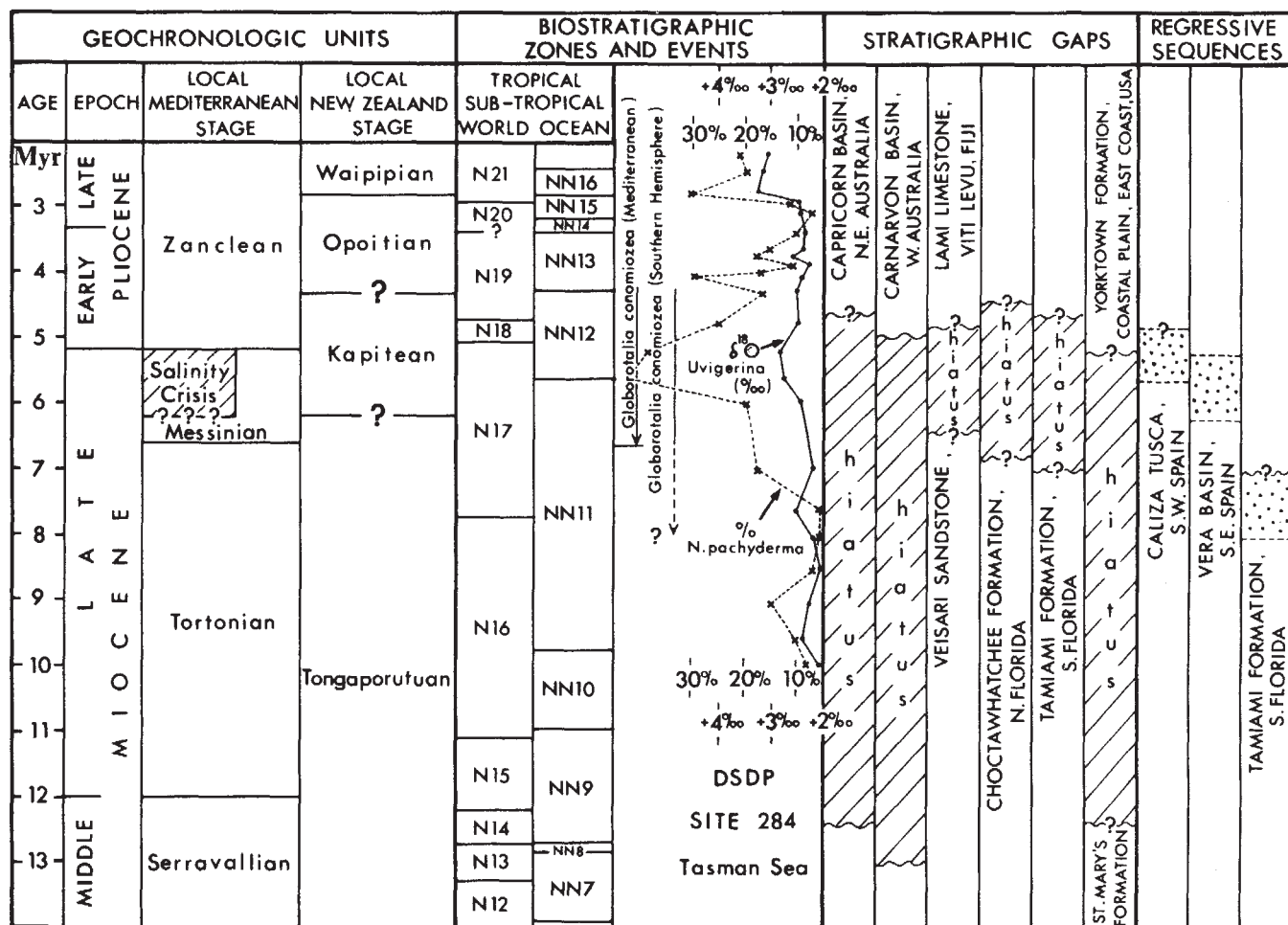


Fig. 1 The Miocene/Pliocene stratigraphic interval with position of stage boundaries and the Mediterranean salinity crisis relative to world ocean biostratigraphic zones, various climatic events, erosional gaps in shallow-water formations and regressive sequences discussed in the text.

oscillations. We examine here the evidence for eustatic change without implying that it was the only major physical process associated with the Messinian Salinity Crisis or that it was necessarily the most important.

The Gibraltar Sill has a depth of 320 m at present. This is sufficiently shallow to keep cold, deep Atlantic waters out of the Mediterranean, but not so shallow as to prevent the reflux of a higher salinity (38‰) intermediate water mass as a consequence of excess evaporation over river run off and precipitation in the Mediterranean region<sup>11</sup>. Despite its great depth (> 3,000 m in places) the modern Mediterranean is thermospheric, that is, its bottom waters are everywhere warmer than 10 °C. Oceanic bottom waters on the other hand are psychrospheric, that is, 10 °C or less at depth. Some sediments of Eocene to Middle Miocene (Serravallian) age in the Mediterranean area contain psychrospheric ostracodes which, according to Benson<sup>12</sup>, have their upper depth limits closer to the 8 °C than to 10 °C isotherm while those of Late Miocene (Tortonian and Messinian) age do not<sup>13</sup>. The marine sediments of latest Miocene age can therefore be regarded as deposits of a thermospheric sea.

The salinity crisis could not have been initiated until the Iberian Portal became shallow enough to cause the Mediterranean progressively to concentrate salt as a consequence of ending the present-day type of brine reflux. Once this stage was reached communication through the Iberian Portal would essentially be one way, and the balance between rate of supply and the rate of evaporation would dictate when and if drawdown would start in the now isolated Mediterranean<sup>14</sup>. Normal or near normal marine

planktonic faunas would perhaps persist for some time until increasing salinity led to their ultimate disappearance as when the Red Sea became isolated from the Gulf of Aden at 22,000 BP during the last ice age<sup>15,16</sup>.

Before the total isolation of the Mediterranean, the Iberian Portal might have been a wide, shallow channel through which a permanent current flowed from west to east. One of us (W.B.F.R.) suggests that in response to eventual drawdown of the Mediterranean, the portal may have evolved into a series of interconnected marine waterways lying at different base levels relative to the Atlantic and separated by cataracts. Evaporation along the route through the portal may have been minimal accounting for the observation of H. Dronkert in the Vera Basin of southern Spain of shallow-water corals and bioherms sandwiched between Messinian gypsum with bird tracks on bedding planes<sup>17</sup> and deep-water pelagic marls and turbidites containing *Globorotalia conomiozea*<sup>18</sup>. Parts of the cascading passageway would eventually have been subjected to tectonic modification and subaerial erosion before its remnants were filled with Pliocene or later marine sediments.

There are several supposedly continuous Miocene/Pliocene marine successions in the Mediterranean region (Chelif Basin, Algeria; the circum-Troodos area of Cyprus; and the central and western parts of Crete) which on present evidence do not seem to support the deep basin desiccation hypothesis as formulated after the 1970 deep-sea drilling cruise<sup>2</sup> and later sustained by further drilling in 1975 (ref. 19). Of the land areas mentioned above, the first two are inadequately known and the third is not noted for the quality of its exposures and faunas at the critical

level. These anomalies require further careful investigation as does the succession recently described by Montenat *et al.*<sup>20</sup> in the Vera Basin, Spain.

The flow of normal marine water through the shallow Iberian Portal envisaged above, would have been affected by changes in sea level. A substantial global fall might have led to a total restriction of the Mediterranean and to consequent desiccation<sup>21,22</sup>. Thus, it is pertinent to examine the available sedimentary and faunal evidence for Late Miocene alterations in global sea level.

The deposition of shallow-water marine carbonates (inter-tidal, subtidal, platform and shelf facies, etc.) was a normal and continuing process within the circumtropical zone throughout most of Cenozoic time, fossiliferous limestones of Palaeocene to Middle Miocene age being common in the central Americas, Mediterranean and Indo-Pacific regions. Upper Miocene limestones are, however, rare everywhere<sup>23,24</sup>, and there seem to be no described shallow-water carbonates spanning the Miocene/Pliocene boundary. Even in the Pacific (for example, Bikini and Eniwetok atolls) where continuous deposition across this boundary was once thought to have occurred<sup>25,26</sup>, reappraisal of the foraminiferal faunas has shown that assemblages characteristic of the latest Miocene are absent<sup>23</sup>. In the Capricorn Basin of NE Australia, a faunal and physical break has been demonstrated between shallow-water carbonates of N14 and N19 age in the Wreck Island Bore<sup>27</sup>, while in the off-shore part of the Carnarvon Basin, western Australia, a major disconformity separates the shallow-water N13 carbonates penetrated in the Tryal Rocks No. 1 bore from the overlying limestones of N18 age<sup>28</sup>. The most comprehensive review of the stratigraphical succession in Indonesia<sup>29</sup> includes no examples of limestones spanning the Miocene/Pliocene boundary. Baumann<sup>30</sup> has, however, recently published a diagram (ref. 30, Fig. 2) showing this boundary within the Karren Limestone of NE Java but without adducing any supporting data. There is, in fact, no faunal evidence that any part of the limestone is Miocene, and, if it were, it would still be necessary to prove continuous deposition across the Miocene/Pliocene boundary in order to affect the present argument. A recently completed study of the Miocene/Pliocene succession in Viti Levu, Fiji (Adams and Rodda; in preparation) proves that an angular unconformity separates N17 from N18/19 sediments. A clearly recognisable stratigraphic hiatus exists on the Sea of Japan side of Honshu (Lloyd H. Burckle, personal communications). The Caliza Tosca, a shallow-water limestone some 60 m thick in Andalusia, Spain, is generally regarded<sup>31</sup> as Messinian in age and therefore coeval with the Mediterranean evaporites. This age determination, however, is based on stratigraphic position and the absence from a critical sample (C12) of *Discoaster quinqueramus*, rather than on positive evidence, and is therefore questionable. There seems to be no palaeontological proof that it is not early Messinian, that is, pre-evaporitic. The thick succession of carbonates and evaporites which make up the Bahamas Platform in the West Indies extends from the Recent down at least to the Lower Cretaceous, but the Andros deep well (14,585 feet) yielded no evidence for the position of the Miocene/Pliocene boundary<sup>32</sup> and none for or against continuous carbonate deposition throughout the Late Miocene.

Examination of the Upper Miocene—Lower Pliocene carbonate-clastic sequence in northern Florida, USA<sup>33</sup> shows a pronounced erosional unconformity between the Choctawhatchee Formation (N17) and the overlying N19 sediments. In southern Florida, the Tamiami Formation, a shallow water carbonate unit, is broken by an unconformity separating N17 from N19 sediments<sup>34</sup>. A similar unconformity occurs in the coastal plain of Maryland, Delaware and New Jersey separating yellow-white glauconitic sands and silts of the Yorktown Formation of latest

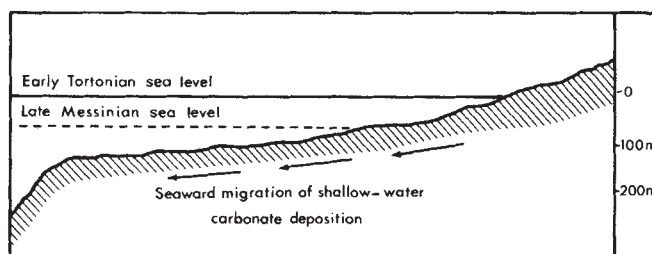


Fig. 2 Hypothetical migration of the locus of shallow-water carbonate deposition towards the shelf-edge during the suggested Messinian age eustatic sea-level fall of 40–70 m in the world ocean.

Miocene to Pliocene (?) age from bluish-grey clay belonging to the St Mary's Formation of Middle Miocene age<sup>35,36</sup>.

Since shallow-water deposits of the type we have been referring to are deposited within the photic zone, a fall in the level of the world ocean would ensure that their area of deposition migrated towards the edge of the continental shelves. The greater the eustatic drop, the farther the zone of deposition would move. Nearshore limestones of pre-Messinian age would either be eroded or buried by clastic sediments deposited in brackish or freshwater milieux. New limestones would form farther away from the old coast lines (Fig. 2) and, where sedimentation was continuous, would rest on somewhat deeper-water sediments of Tortonian age. Changes in sedimentation would occur across the continental shelves of the entire world, and the lowering of sea level would be reflected in their faunas. Only where considerable uplift has occurred could such limestone be found on the land surface today.

The possibility of a Late Miocene eustatic change is also suggested by the facies changes in ostracode faunas and sediments of the Caribbean and on the Atlantic Coast of the United States. The ostracodes of these regions are well known (refs 37, 38). Of special interest are those from Blow's zones N13 to N21 from Cuba, Hispanola, Puerto Rico and Trinidad, although faunas of the same age and latitude are known from Central America. In almost every case the palaeoenvironmental succession indicates a rapid shallowing upward from N16, often followed by a hiatus, then by a deepening. The exceptions occur in Panama, where the deposition of the shallow Gatun Formation was followed by continental deposition, and in Costa Rica, where shallowing was slow and continuous. Often the stratigraphic control of these shallow beds is through inference or benthic microfaunal successions as planktonics are rare. In Trinidad, the Springvale Formation is a clay-glaucconitic sand mixture; carbonates of N17 age are rare, stratigraphically doubtful or absent. The Jamaican section is carbonate, but deep; it nevertheless shows noticeable shallowing during the Late Miocene.

There is good evidence from widely separated areas that marked faunal and floral changes occurred during the late Miocene. In California<sup>39,40,41</sup>, Alaska<sup>42</sup>, the Atlantic<sup>43</sup>, the Pacific<sup>44</sup>, the Antarctic and southern oceans where foraminiferal assemblages and the isotopic composition have suggested<sup>45,46</sup> both cooling and a drop in sea level. There is also evidence from the land floras from a cooling in the Late Miocene of NW America<sup>47</sup>.

The benthic foraminiferal fauna of the south Florida Tamiami Formation, underlying the N17–N19 unconformity indicates a shoaling environment which became increasingly restrictive with time<sup>34</sup>. This fauna is replaced by a lacustrine diatom assemblage at the Miocene/Pliocene boundary. Rocks of the same age in northern Florida also indicate a prograding succession of events that may also be due to a Late Miocene drop in sea level<sup>33</sup>.

Changes in the frequency<sup>42,48</sup>, coiling direction<sup>49,51</sup> and  $\delta^{18}\text{O}$  composition of planktonic foraminifera indicate an influx of cold water into the New Zealand area during a



stratigraphic interval known locally as the Kapitean stage. Reinterpretation of the palaeomagnetic stratigraphy on the Mangapoike and Blind River sections<sup>52</sup> suggests that regressive sediments and discontinuities in the Kapitean are time correlative<sup>5</sup> with the Messinian stratotype of Sicily. Coiling ratios from strata off NW Australia referable to zone N18 also indicate cooling of the higher latitude seas at the close of the Late Miocene.

This cooling and accompanying regression have been attributed to an expansion of Antarctic glaciation leading to a fall in sea level of perhaps 50–70 m, recognisable during the later part of the Andalusian<sup>31</sup> and consistent with the magnitude of ice volume change recorded in the  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  isotopic data on *Uvigerina* from DSDP Site 284, south Pacific<sup>45</sup>.

A substantial fall in the level of the world ocean, that is more than about 50 m, would be sufficient to convert some shallow shelf areas into inland lakes. The Sea of Japan would be isolated by a fall of 70 m, and it would be interesting to learn whether evidence for freshwater sediments of late Miocene age has been obtained from drill holes in this area.

The lowering of global sea level could have been the event which closed off the Iberian Portal, leading to the Mediterranean isolation and eventual desiccation<sup>10,53</sup>. Conversely, the extraction of 6% of the dissolved salts in the world ocean brought about by the Mediterranean desiccation<sup>8</sup> could have induced the late Miocene glaciation by lowering high latitude salinity sufficient to raise the freezing point of seawater. Other possible effects of the massive salt extraction are the abrupt change in the percentage carbonate (relating to the dissolution of calcareous tests) occurring in drill holes in the Pacific, Atlantic and Caribbean areas at a stratigraphic level very close to the boundary between Upper Miocene and Lower Pliocene sediments<sup>9</sup> and the apparent absence of even shallow-water limestones at that time<sup>24</sup>. It may be more probable, however, that the onset of glaciation was independent of Mediterranean events, and high latitude cooling was the principal mechanism to cause areas of carbonate deposition to migrate away from the land.

Precise dating of these numerous events of the Late Miocene is fraught with difficulties because of the short period of time involved and the consequent imprecision of stratigraphical correlation using palaeobiological methods. A general lowering of sea level, however, of the order of 40–70 m would certainly have had a dramatic effect on the sediments and faunas of the continental shelves. Evidence of this should be preserved in bore-hole records, the investigation of which could reveal the duration and order of magnitude of any change. The Tortonian/Messinian eustatic-fall hypothesis is therefore eminently testable and can be verified or refuted by stratigraphers with access to drilling records in such areas as the Gulf of Mexico, China Sea and Persian Gulf.

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## Evidence for a sensitising pigment in fly photoreceptors

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*Many photoreceptor cells in invertebrates have a dual-peak spectral sensitivity. Evidence is presented that in fly photoreceptors the ultraviolet peak is due to a photostable pigment that absorbs light quanta and transfers the energy to the blue-absorbing visual pigment.*

THE primary process induced by absorption of a light quantum in a visual-pigment molecule is the isomerisation of the chromophore retinaldehyde from the 11-*cis* to the all-*trans* form<sup>1</sup>. Subsequent dark reactions finally lead to an excitation of the receptor cell. We present here evidence that in addition to this direct interaction between light and visual pigment,

another process can take place—the light quantum can be absorbed by an accessory pigment which then transfers the energy to the visual pigment. The photostable accessory pigment is therefore acting as a 'sensitising pigment'. Sensitisation is known in photography where it is used to extend the spectral sensitivity of silver halides<sup>2</sup>. In plant cells also, photostable accessory pigments (for example carotene) absorb light and transfer the energy to the effector pigment, chlorophyll *a*, for use in photosynthesis<sup>3</sup>.

## Fly photoreceptors

In the compound eye of flies six of the eight receptor cells in each ommatidium have a receptor-potential action-spectrum with two maxima of similar height, one close to 500 nm, the other in the near ultraviolet, close to 360 nm (refs 4, 5). Dual-peak sensitivity of this type cannot be explained on the basis of extinction spectra of known rhodopsins: these pigments have only a small peak at shorter wavelengths, of less than 25% of the maximum ( $\beta$ -peak)<sup>6</sup>.

On the basis of electrophysiological experiments using selective chromatic adaptation, Burkhardt<sup>4</sup> concluded that there was only one visual pigment present in each photoreceptor. Later data were thought to suggest the existence of two different rhodopsins and metarhodopsins in one and the same receptor cell<sup>5,7</sup>, but the most recent results<sup>8</sup> again support the view that there is only one visual pigment in these receptors.

Receptor-potential recordings are not a direct method of characterising visual pigments, for two reasons, first, it is not possible to exclude electrical interactions between different receptor cells with any certainty; and second, it is not possible to discriminate between effects of visual pigment *per se* and effects of accessory pigments which could modify the properties of photoreceptors in several ways<sup>9</sup>.

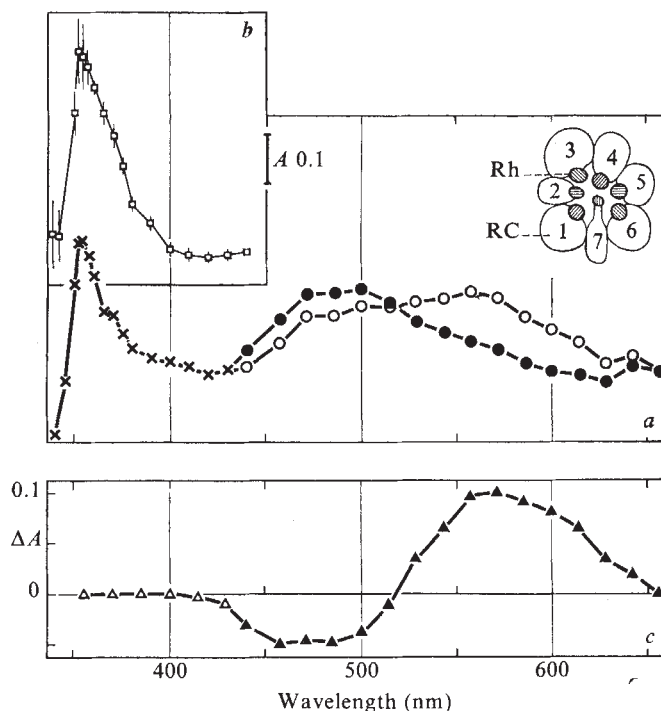
Microspectrophotometry provides more direct data on visual pigments. Difference spectra in receptors 1 to 6 have been measured in the visible range ( $\lambda = 400\text{--}700\text{ nm}$ )<sup>10–13</sup>. These data (from *Calliphora*) show that there is only one isosbestic point at 510 nm, irrespective of which combination of two wavelengths of the spectrum is used for the conversion of rhodopsin into (dark-stable) metarhodopsin and *vice versa*. The difference spectrum is consistent with the existence of a rhodopsin with peak absorption at 490 nm, which nicely fits the spectral sensitivity in the visible, and a metarhodopsin with peak absorption at 580 nm. The fixed isosbestic point also indicates that there should be only one visual pigment in these receptors. But, it does not exclude with certainty the existence of a second visual pigment, since this could be non-absorbing in the limited spectral range investigated so far, or its spectrum could strongly overlap that of its metarhodopsin and hence exhibit a null difference spectrum. Results from microspectrophotometry so far give no explanation for the high ultraviolet sensitivity measured electrophysiologically.

Since there must be absorption of light in the ultraviolet if the receptor cells show high ultraviolet sensitivity, we decided to record not only the difference spectrum but also an absolute spectrum and to extend the measurements into the ultraviolet spectral range.

## Microspectrophotometry

For microspectrophotometry, dipteran photoreceptors have the advantage that—in contrast to the situation in for example, locust or bee—the rhabdomeres in each ommatidium are not fused together but are separated from each other over their whole length, so that they act as individual light guides. It is therefore possible to measure the extinction of individual rhabdomeres in an eye cup preparation as was first done by Langer<sup>14</sup>. These structures are only 1–2  $\mu\text{m}$  in diameter, so waveguide effects slightly modify the measured extinction spectra; however, this minor effect<sup>15</sup> is within the limits of error of our data and no correction was applied.

Figure 1a shows two extinction spectra of type  $R_{1-6}$  receptors

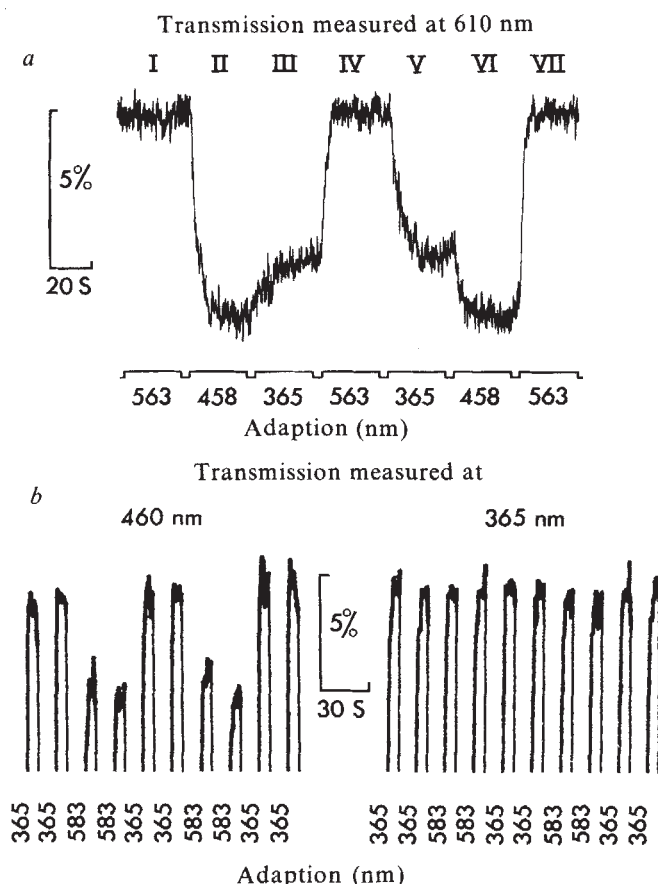


**Fig. 1** *a* and *b*, Extinction spectrum of rhabdomeres type  $R_{1-6}$  (*Musca* ♀, eye-cup preparation of a white eyed mutant) as measured with a technique similar to that of Langer<sup>14</sup>, but using a single beam instrument (Leitz MPV2, with Zeiss ultrafluor-optics, selected for minimal chromatic aberration). During measurements in the spectral range from 440–660 nm the preparation was illuminated with strong adapting lights of 365 nm (○) or 583 nm (●) at regular intervals, in order to maintain a stable equilibrium of rhodopsin/metarhodopsin. During adaptation the photomultiplier was protected by a mechanical shutter. Since other experiments showed that there is only a slight change or no change in extinction for wavelengths shorter than 430 nm after ultraviolet or orange adaptation, the spectrum in this range was measured without adaptation (×). ○, ●, ×, Data from rhabdomeres no. 2, 3 and 4 (measured together) of one ommatidium. In order to get a more accurate spectrum in the ultraviolet, rhabdomeres (no. 4 and 5) from 5 ommatidia of another eye were measured and the mean spectrum of all 6 ommatidia calculated (●); the given standard error was obtained after shifting the spectra along the ordinate to coincide at  $\lambda = 440\text{ nm}$ . Zero extinction is not defined in this kind of measurement, since the measuring beam passes the convergent cornea-lens of the ommatidium, whereas the reference beam does not. The cornea was shown to transmit uniformly in the spectral range analysed and fluorescence did not affect the measured spectra. Temperature of the preparations was 6–8 °C. Inset: cross section through ommatidium indicating receptor cells RC and rhabdomeres Rh. *c*, Difference of spectra from *a* (▲). Additional measurements at the short wavelengths (△) were carried out in the same rhabdomeres. Other rhabdomeres  $R_{1-6}$  sometimes showed reproducible small negative differences in the ultraviolet.

from a white-eyed *Musca* ommatidium, adapted with orange ( $\lambda = 583\text{ nm}$ ) and ultraviolet ( $\lambda = 365\text{ nm}$ ) light, respectively. It is obvious that there is a high extinction in the ultraviolet, which is confirmed by data from five more ommatidia (Fig. 1*b*). A second maximum occurs either near 500 nm or near 560 nm, respectively, depending on the pre-adapting light. The difference (Fig. 1*c*) between the two curves of Fig. 1*a* represents the difference spectrum between metarhodopsin (M 580) and rhodopsin (R 490), which corresponds to that obtained in intact animals<sup>11</sup> and in wild-type flies (*Calliphora*)<sup>13</sup>. We have confirmed that the difference spectrum in *Musca* changes only in amplitude, not in shape, when the wavelengths used for shifting the pigment from one stable state (R 490) to the other (M 580) are changed, and we have extended the measurements into the ultraviolet.

In spite of the fact that there is a high absorption in the ultraviolet (Fig. 1*a, b*) and that ultraviolet light induces a shift from rhodopsin to metarhodopsin, there is no conspicuous





**Fig. 2** *a*, Transmission of rhabdomeres no. 2+3+4 (*Musca* ♀, white-eyed) measured at 610 nm. Adapting lights of different colours were used as indicated. Appropriate edge filters prevented transmission of adapting light to the photomultiplier. *b*, Transmission measured in the blue (460 nm) and ultraviolet (365 nm) following various adapting lights as indicated. In contrast to Fig. 2*a* the adapting light was switched off during the measurement.

decrease in the ultraviolet extinction. As this result was unexpected we checked directly whether the blue and ultraviolet lights shift the pigment to the same or to two different metarhodopsins, both absorbing in the red. The temporal change in metarhodopsin concentration was monitored by measuring transmission at 610 nm: the higher the transmission, the lower the metarhodopsin concentration (Fig. 2*a*).

After an equilibrating pre-adaptation with 563-nm light which shifts the pigment to the rhodopsin state, both blue (458 nm, Fig. 2*a* I→II) and ultraviolet (365 nm, Fig. 2*a* IV→V) lights shift the pigment back to metarhodopsin, as indicated by the reduction in transmission at 610 nm. The equilibrating blue light shifts more than the equilibrating ultraviolet light.

In fact, ultraviolet light following blue light (Fig. 2*a* II→III) leads to a conspicuous increase in transmission at 610 nm. If there had been two different red light-absorbing metarhodopsins in the one photoreceptor, this ultraviolet stimulus should have led to a further decrease in transmitted red light, due to the formation of the second metarhodopsin. The observed increase indicates that, during ultraviolet illumination, a new equilibrium between rhodopsin and metarhodopsin is created which yields less metarhodopsin than does blue illumination. These results are evidence that the metarhodopsin formed by ultraviolet light is the same as that formed by blue light.

Figure 2*b* illustrates directly the fact that ultraviolet light following orange (583 nm) light reduces the rhodopsin concentration, as shown by an increase in transmission in the blue (460 nm), despite the fact that there is no change or only a slight change, in ultraviolet absorption in equivalent conditions (Fig. 2*b*).

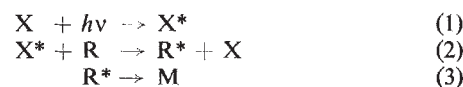
The results obtained with microspectrophotometry can be interpreted in at least three ways. (1) There is a second visual pigment that absorbs in the ultraviolet, and has a metarhodopsin also absorbing in the ultraviolet. Their strongly overlapping spectra would explain the odd absence of extinction change in the ultraviolet (Fig. 1). (The shift of pigment to M 580 by ultraviolet illumination is then due to the  $\beta$  peak of R 490). (2) The rhodopsin and metarhodopsin are unusual insofar as they both absorb strongly in the ultraviolet. (3) There is a photostable pigment absorbing in the ultraviolet and transferring the energy to the rhodopsin. It then acts as a sensitising pigment.

One possible mechanism of energy transfer is by means of dipole-dipole interactions (case 3*a*), according to Förster's theory<sup>16</sup>. In this case the photostable pigment is called an 'antenna' pigment, as in photosynthesis. Other kinds of energy transfer are also possible, for example, a photostable chromophore could be attached to the rhodopsin molecule, giving rise to a high ultraviolet absorption in rhodopsin (as well as in metarhodopsin) (case 3*b*). Such a mechanism, in a way, is rather similar to the situation in photographic sensitisation, in which a dye must be adsorbed on to the silver halides in order to act as a sensitiser<sup>2</sup>. The trivial case in which light quanta, emitted due to fluorescence of a photostable substance, are reabsorbed by the rhodopsin seems rather unlikely because of the low efficiency of this process within a rhabdomere.

According to electrophysiological data, hypothesis (1) is improbable, since intracellular recordings made in cells 1–6 under selective chromatic adaptation have revealed that blue-light adaptation depresses sensitivity in the blue and the ultraviolet to the same extent and vice versa<sup>4,8</sup>. We present here experiments that exclude hypothesis (2) as well as (1) and give evidence that hypothesis (3) is realised.

## Evidence for a sensitising pigment

The sensitising-pigment hypothesis can be formulated as follows:



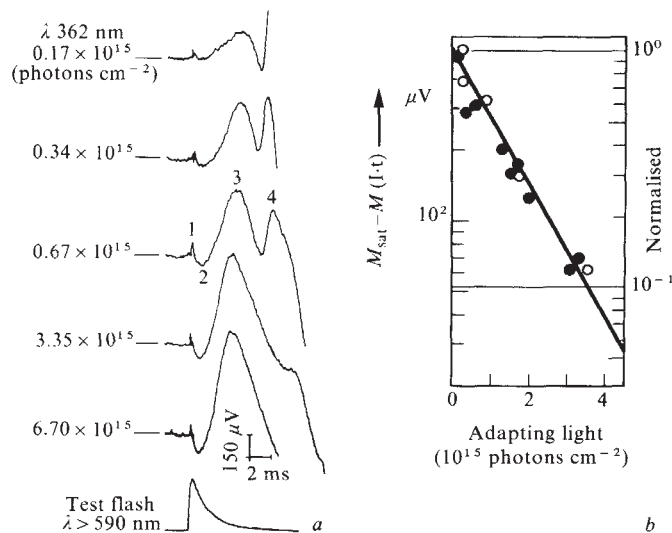
Due to the absorption of a light quantum, the unspecified molecule  $X$  is converted into the excited state  $X^*$  (equation 1). In a secondary process it then may interact with a rhodopsin molecule  $R$ , converting it into an excited state  $R^*$  (equation 2), which finally leads to metarhodopsin  $M$  (equation 3). Alternatively, the molecule  $X^*$  may lose the extra energy by fluorescing



or by suffering deactivating collisions with other molecules. In the latter case the energy is dissipated as heat. If the energy transfer occurs by means of a dipole-dipole interaction (case 3*a* as discussed above), the concentration of the molecules present determines whether the secondary process occurs according to equations (2) and (3) or to equation (4), assuming that the lifetime of the excited state is sufficiently short. This is because these concentrations affect the probability that a molecule  $X^*$  meets a molecule  $R$ <sup>17</sup>. The relationship between the percentage of rhodopsin still present and the number of bleaching quanta should be a decreasing exponential for blue as well as for ultraviolet quanta, as long as the concentration of  $R$  and  $X$  is comparatively high, since the kinetics in both cases corresponds to classical first-order photochemical bleaching.

But, if we reduce sufficiently the concentration of  $X$  and/or  $R$  we expect to observe the following two phenomena: (1) The ratio of the number of ultraviolet quanta to blue quanta necessary to shift a given percentage of rhodopsin into metarhodopsin must be increased with a sufficiently decreased concentration of  $R$  and/or  $X$ . (2) An exponential decrease of rhodopsin concentration due to bleaching is still expected for blue light,





**Fig. 3** *a*, Initial part of electroretinograms on a rapid time scale obtained with high-energy orange test flashes of constant intensity from a white-eyed *Drosophila* raised on Sang's synthetic diet medium<sup>24</sup> with 2.4 mg per 100 ml  $\beta$ -carotene added (8 mg per 100  $\pm$  100%). The flash light-source (photographic flash, Braun F900) was used in conjunction with a Schott OG 590 edge filter and a Schott KG1 heat filter. The energy of the flash when using a monochromatic 584-nm interference filter (Schott, Depal) together with KG1 heat filter, was  $5.4 \times 10^{15}$  photons  $\text{cm}^{-2}$  at the level of the preparation. Before each measurement the eye was given two orange flashes that shift the pigment to the rhodopsin state. After 1 min in the dark the amount of monochromatic 362 nm ultraviolet light (Schott, uv-pil) indicated on the left of each trace was applied. After 1 min in the dark the orange test flash (590 nm) of constant intensity was given, inducing the five responses shown. Each orange flash elicits a mass response composed of several components indicated by numbers on the third trace: 1, stimulus artefact, indicating the onset of the flash; 2 and 3, biphasic fast photovoltage proportional to the concentration of pigment in the metarhodopsin state. This response called the M-potential, can be subdivided into a negative phase (2) and a positive phase (3)<sup>18</sup>. 4, 'on' transient of the ERG which arises from the response of the second-order neurones. *b*, The ordinate gives the difference between the peak M-potential amplitude following saturating ultraviolet light ( $M_{\text{sat}}$ , bottom M-potential trace in *a*) and the M-potential amplitude  $M(I-t)$  following various amounts ( $I-t$ ) of adapting 362-nm ultraviolet (●) and 473-nm blue (○) light. The curve actually represents the decay of rhodopsin concentration as a function of the amount of adapting light.

but no longer for ultraviolet light. This is because the concentration of rhodopsin, further reduced by an increasing number of absorbed ultraviolet quanta, reduces the efficiency or energy transfer between  $X^*$  and R. The kinetics can then no longer be first order.

To test these predictions we measured the concentrations of fly visual pigments by an electrophysiological technique. This independent and sensitive method has the advantage of using intact flies whereas the microspectrophotometric measurements were carried out with eyecup preparations. The pigment conversion in receptors  $R_{1-6}$  was measured by a fast photovoltage, the M-potential<sup>18</sup>, which is a response proportional to the metarhodopsin concentration [M] in  $R_{1-6}$ , as shown by the following evidence. (1) The action spectrum of the M-potential corresponds to the metarhodopsin extinction spectrum of receptors  $R_{1-6}$  (ref. 18). (2) No M-potential has been observed in mutants lacking receptors  $R_{142}$  (S. R. Grabowski and W. L. Pak, personal communication). (3) In a dark-adapted eye, the M-potential amplitude increases exponentially with the amount of incident blue quanta and reaches saturation at the same level at which metarhodopsin formation saturates (Minke *et al.*, unpublished).

M-potentials were recorded from a white-eyed *Drosophila* with an approximately normal rhodopsin concentration, in

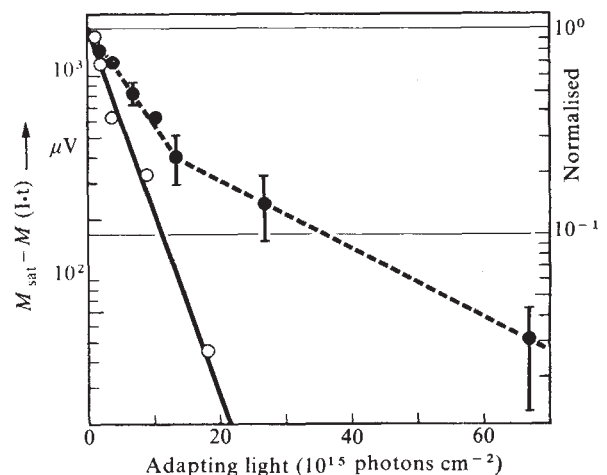
response to a strong orange test flash, following various amounts of 362-nm ultraviolet pre-adapting light (Fig. 3a). Figure 3b shows a plot of the difference between the M-potential from rhodopsin to metarhodopsin. This experiment is a control amplitude measured after an equilibrating ultraviolet illumination and the M-potential amplitude measured after different amounts of pre-adapting ultraviolet light. This method of presentation actually illustrates the decay of rhodopsin concentration [R] with increasing ultraviolet illumination, since  $[R] = 1 - [M]$ .

Equivalent data for 473-nm blue adaptation are also included in Fig. 3b. The data show that the rhodopsin concentration decreases exponentially with the amount of adapting blue or ultraviolet light. In this case the 362-nm ultraviolet light is as efficient as the 473-nm blue light in shifting the visual pigment from rhodopsin to metarhodopsin. This experiment is a control experiment for normal flies. In the critical experiment we need flies with rhabdomeres in which the concentrations of rhodopsin and/or the presumed sensitising pigment are reduced.

The concentrations of rhodopsin within the rhabdomeres can be reduced by raising flies in a vitamin A-deficient medium<sup>12,19,21,22</sup>. Figure 4 shows the decay of the rhodopsin concentration, again measured by the M-potential, as a function of the amount of adapting 473-nm blue and 362-nm ultraviolet light, measured in a vitamin A-deprived fly. First, it seems that about twice as many ultraviolet quanta as blue quanta are now necessary to produce a criterion M-potential of 50% of the maximum (in three other experiments two to nine times as many ultraviolet quanta were necessary). Second, the dependence of the rhodopsin concentration on the amount of adapting ultraviolet light is no longer exponential. This is in contrast to the dependence on blue-adapting light (measured in the same fly), which remains exponential.

Thus in vitamin A-deprived flies, the effectiveness for converting rhodopsin into metarhodopsin is selectively reduced in the ultraviolet. This is in agreement with the result that the sensitivity of the receptors as determined with the ERG in vitamin A-deprived flies is selectively reduced in the ultraviolet<sup>19,23</sup>. This finding, as well as the fact that the kinetics of the decrease of rhodopsin concentration under ultraviolet illumination no longer corresponds to a first-order reaction, fits the predictions of the 'antenna' pigment model.

**Fig. 4** Concentration of pigment in the rhodopsin state, as measured by the M potential, in response to the orange test flashes of constant intensity, as a function of the amount of adapting 362-nm ultraviolet (●) and 473-nm blue (○) light. All the measurements were from a single white-eyed *Drosophila* raised on vitamin-A-deficient medium (Sang's synthetic diet medium with 0.8 mg per 100 ml  $\beta$ -carotene). The vertical bars are standard deviations of the mean calculated from three or four measurements. Most of the points without bars are the average of two measurements.



Our results are not compatible with the hypothesis of a second rhodopsin-metarhodopsin, absorbing in the ultraviolet (model 1). This model would predict neither the high efficiency of ultraviolet quanta for the production of M 580, as shown in Fig. 3b, nor the modifications seen in flies raised on vitamin A-deprived media (Fig. 4). An unusual rhodopsin (model 2) would also be unable to generate the modifications observed in vitamin A-deprived flies. Although the antenna-pigment model is sufficient to explain the experimental results, this kind of sensitisation is not necessarily the only possible one. With several special assumptions, a mechanism of energy transfer according to case 3b, as mentioned above, could also be realised. There is some evidence that the absorption in the ultraviolet is due to a derivative of vitamin A (J. Schwemer, personal communication). If this substance is attached to all rhodopsin molecules as a second chromophore only at sufficiently high vitamin A concentrations, then at lower vitamin A concentrations, two populations of rhodopsin will be present: one with a high ultraviolet absorption due to the second chromophore, and another which lacks the second chromophore and therefore has only low ultraviolet absorption (due to the low  $\beta$  peak of the rhodopsin). The kinetics of the decay of both rhodopsins will be the same if blue light is used for bleaching. There will be two different decay rates, however, if ultraviolet light is used, since the absorption coefficients of the two substances differ in the ultraviolet. This is also in accordance with Fig. 4 (ultraviolet illumination), since the deviation from the pure exponential can be interpreted as being due to the sum of two exponentials with different decay constants.

We conclude that the photoreceptors 1-6 in the fly achieve their high ultraviolet sensitivity thanks to an accessory photostable pigment that acts as a sensitiser for rhodopsin. The mechanism of the energy transfer has still to be worked out in detail. The functional consequence of the sensitising pigment for

the photoreceptors is clear—it broadens the spectral range of the visual pigment and thus increases absolute sensitivity.

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## Nucleotide sequences of 5'-terminal ribosome-protected initiation regions from two reovirus messages

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*Sequences for two reovirus ribosome-protected fragments are presented. Comparison with four other reovirus initiation sites reveals only two common features: the 5'-terminal sequence m7GpppG<sup>m</sup>CUA, and (located 15-33 nucleotides from the cap) the sequence AUGG. Both the cap and the AUG codon are included in the 40S-ribosome protected region from all six reovirus messages.*

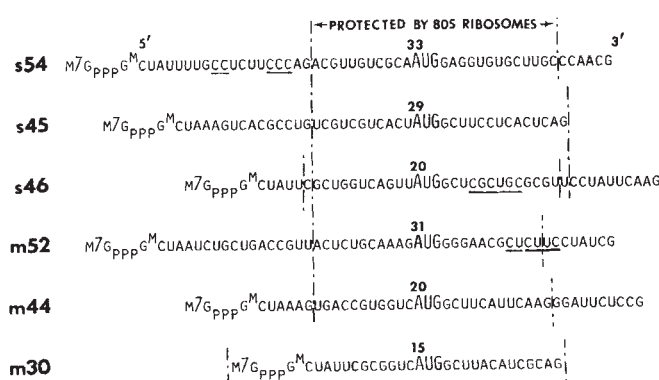
THERE has been considerable speculation about what features in mRNA direct attachment of ribosomes to the sites where translation will be initiated. Although rather sophisticated hypotheses have been advanced for messenger-ribosome interactions in prokaryotic systems<sup>1,2</sup>, deductions about eukaryotic mechanisms have been limited by the absence of detailed information concerning the initiation sites from a variety of eukaryotic messages. The sequence extending from the 5' capped terminus up to and beyond the AUG initiation triplet was reported for one of the genome segments from brome mosaic virus<sup>3</sup> and, more recently, for rabbit  $\beta$ -globin mRNA<sup>4,5</sup>. We have previously described the nucleotide sequences of ribosome-protected regions from four reovirus messenger RNAs<sup>6,7</sup>. We present here sequences for two additional reovirus ribosome binding sites. All six ribosome-protected frag-

ments from reovirus mRNA include the 5'-terminal methylated cap, which has been shown to strongly promote *in vitro* translation of various eukaryotic messages<sup>8</sup>.

### Isolation of two ribosome-protected fragments

Reovirus mRNAs, labelled with  $\alpha$ -<sup>32</sup>P-ribonucleoside triphosphate precursors, were synthesised *in vitro* by virion-associated enzymes, in conditions described previously<sup>6</sup>. The *in vitro* transcription reaction catalysed by reovirus cores generates 10 presumably monocistronic messenger species which can be fractionated by sucrose gradient velocity sedimentation into small, medium and large size classes<sup>9</sup>. In this study, the small size class (*s*-RNA), consisting of four messenger species, was incubated in a wheat germ extract in conditions permitting formation of both 40S and 80S initiation complexes<sup>6,7</sup>. After addition of T<sub>1</sub> RNase to hydrolyse the exposed regions of the RNA, the samples were sedimented through glycerol gradients<sup>6,7</sup>. The <sup>32</sup>P-labelled material recovered from 40S ribosomal complexes consisted of a mixture of three cap-containing fragments, which were readily resolved by polyacrylamide gel electrophoresis followed by two-dimensional homochromatography<sup>7</sup>. Nucleotide sequences were previously determined for two of these *s*-RNA fragments<sup>7</sup> (s54 and s45 in Fig. 1). The following section describes the sequence of the third fragment, designated s46; that is, a 46-nucleotide fragment





**Fig. 1** Sequences of 5'-terminal ribosome-protected initiation regions from six reovirus mRNAs. An *s* designates fragments derived from the small size class of messenger RNA; *m* designates fragments from medium sized messages. The entire sequence shown for each fragment was protected against  $T_1$  RNase digestion by wheat germ 40S ribosomes. When 40S complexes were digested, instead, with pancreatic RNase, the site of cleavage at the 3' end of the fragment varied (not shown), but the 5' cap structure remained quantitatively protected. Vertical dashed lines show the smaller portion of each fragment that was protected by 80S ribosomes. With each message, we analysed the protected region after digestion of 80S complexes with guanosine-specific  $T_1$  RNase, and also after digestion with pancreatic RNase, which cleaves next to pyrimidine residues: results obtained with the nuclease that cut closer to the AUG triplet were used to construct the diagram above. Data are from refs 6, 7 and this work. Of the sequences shown, fragment m30 is the only one that came from 80S rather than 40S complexes. A 40S-protected capped fragment, about 36-nucleotides long, was fingerprinted and shown to overlap m30, but the complete sequence of the 40S-protected fragment was not determined due to insufficient material. There is an uncertainty in the underlined portion of fragment s54, which might alternatively be CCCUCUCCC. In fragment m52, the orientation of the underlined regions might be reversed, giving the sequence CUUCCU. Four sequences are possible for the six-base underlined region in fragment s46.

from the *s*-RNA class. (This fragment was designated 45R in ref. 7.)

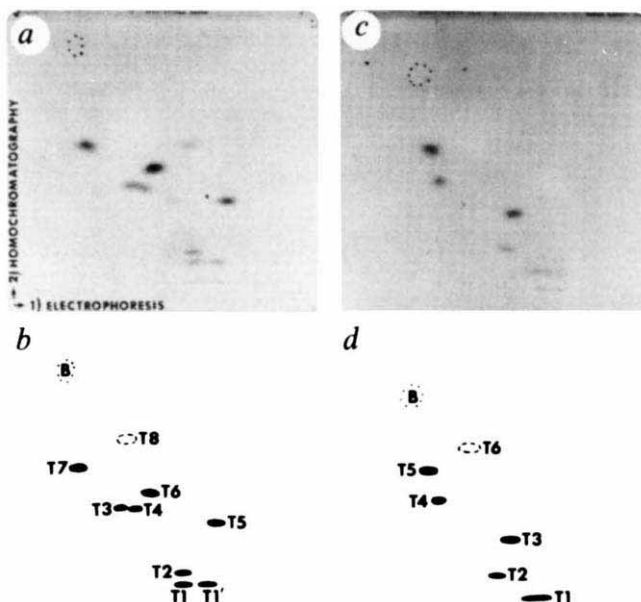
A similar protocol was followed with the medium sized *in vitro* transcripts. The medium size class (*m*-RNA) consists of three non-overlapping, cap-containing protected fragments from 40S initiation complexes<sup>6,10</sup>. Two of these fragments have already been sequenced<sup>6</sup>; they are designated m52 and m44 in Fig. 1. From the third message in the medium size class, a fragment about 36 nucleotides long was recovered from 40S complexes, and a fragment 30 nucleotides long (m30) was protected by 80S complexes<sup>6,10</sup>. Since both the 40S- and 80S-protected fragments from this message retained the 5'-terminal cap, the slightly larger 40S-protected fragment included a few additional 3'-terminal nucleotides. Although our previous sequence determinations were done on both 40S- and 80S-protected fragments from each message (Fig. 1), for the third medium sized message we are reporting the sequence of the 80S-protected fragment m30 only, since the ribosome binding site from this message was recovered in relatively low yield, limiting the manipulations that could be performed.

### Nucleotide sequence of fragment s46

Four preparations of 40S ribosome-protected fragment s46, labelled respectively with  $\alpha$ -<sup>32</sup>P-CTP, -UTP, -GTP or -ATP, were used for these analyses. Use of single-labelled RNA preparations enabled us to exploit nearest neighbour transfer data to help deduce the nucleotide sequences<sup>11</sup>. Aliquots of the purified fragment were subjected to complete  $T_1$  RNase digestion, and to complete pancreatic RNase digestion, followed in each case by two-dimensional fractionation using homochromatography<sup>12</sup>. The experimental procedures used for the present sequence analyses were exactly as described previously<sup>7</sup>. The  $T_1$  RNase limit products, shown in Fig. 2a, were subjected to secondary pancreatic RNase digestion (Fig. 3a) and the composition of each secondary

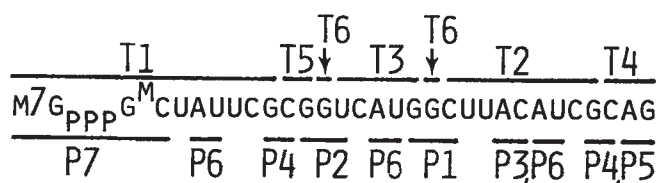
digestion product was determined by alkaline hydrolysis. This provided sufficient information to reveal the sequence of most of the  $T_1$  oligonucleotides, as listed in Fig. 3a. For the large oligonucleotide s46/T2, analysis of the  $U_2$  RNase digestion products (Fig. 3c) was required to establish a unique sequence. Analyses of the primary pancreatic RNase digestion products (panc oligonucleotides) from fragment s46 are summarised in Fig. 3b. Since these data were insufficient to reveal the order of the  $T_1$  oligonucleotides, we subjected fragment s46 to partial  $T_1$  RNase digestion in conditions described previously<sup>7</sup>. Analyses of the two major partial products are summarised in Fig. 3d. Partial product II yielded  $T_1$  oligonucleotides T1, T4, T6 (CUG[G]) and T8. By considering the 5' nucleotides and the 3' nearest neighbour residues for these  $T_1$  oligonucleotides (and taking into account the presence of GGU among the panc oligonucleotides obtained from partial II), the sequence T1-T6-T8-T4 was deduced, as shown at the top of Fig. 3. The sequence of the remainder of the 46-nucleotide fragment can be constructed, as follows. In a complete pancreatic RNase digest of fragment s46, oligonucleotide P1 (AAG) was the only product that terminated with a G residue rather than with a pyrimidine (Fig. 3b); AAG must therefore be derived from the 3' terminus of the protected fragment, which was originally obtained by digestion of initiation complexes with guanosine-specific  $T_1$  RNase. Oligonucleotide T2, the only  $T_1$  RNase product containing AAG, can therefore be placed at the 3' end of fragment s46 (and of the large partial product I). Since T2 begins with a U residue, it must be preceded by T7 (CG[U]), the only  $T_1$  oligonucleotide from partial I that has U as its 3' nearest neighbour. Moving to the 5' end of partial I, panc oligonucleotide P3 (AGU[U]) provides an unambiguous overlap between T4 (already placed at the 3' end of partial II) and T5, the only remaining  $T_1$  oligonucleotide that begins with UU. Thus, partial I begins with T5. Since T5 terminates in G[G], it must be followed by panc oligonucleotide P2 (GGC[U]). The  $T_1$  oligonucleotide that follows T5, therefore, begins with CU; this could be either T6 (CUG) or T3 (CUCG). Since we do not have data which would allow us to order the remaining small oligonucleotides, T3, T6 and T7, there is a 6-base ambiguity (with four alternative sequences) in the coding portion of the sequence deduced for fragment s46. This is shown at the top of Fig. 3.

**Fig. 2** Two-dimensional  $T_1$  RNase fingerprints of  $\alpha$ -<sup>32</sup>P-GTP labelled fragment s46 (*a*, shown diagrammatically in *b*), and fragment m30 (*c*, diagrammatically in *d*). The dashed circle in *b* and *d* shows the position of GMP, which was labelled by  $\alpha$ -<sup>32</sup>P-CTP and -UTP, but not by  $\alpha$ -<sup>32</sup>P-GTP. The capped oligonucleotide T1 in *b* appears as a doublet due to instability of the m7G cap<sup>13</sup>. The dotted circle (*B*) shows the position of the blue marker dye.







a) ANALYSIS OF T<sub>1</sub> RNase PRODUCTS FROM THE 30-NUCLEOTIDE FRAGMENT

| T <sub>1</sub> RNase primary product | Products of secondary digestion with pancreatic RNase |                         |                         |                         | Sequence deduced               |
|--------------------------------------|-------------------------------------------------------|-------------------------|-------------------------|-------------------------|--------------------------------|
|                                      | [α- <sup>32</sup> P]CTP                               | [α- <sup>32</sup> P]UTP | [α- <sup>32</sup> P]GTP | [α- <sup>32</sup> P]ATP |                                |
| m30/T1                               | C, U, cap                                             | AU, cap                 | C, cap                  | U                       | m7Gppp <sup>m</sup> CUAUUCG[C] |
| m30/T2                               | AU, AC, C                                             | AU, C, U                | C                       | U, AC                   | CUUACAUCG[C]                   |
| m30/T3                               | U                                                     | AU                      | AU, G                   | C                       | UCAUG[G]                       |
| m30/T4                               | —                                                     | AC                      | AG                      | C                       | CAG[U]                         |
| m30/T5                               | —                                                     | —                       | G, C                    | —                       | CG[G]                          |
| m30/T6                               | C                                                     | C                       | —                       | —                       | G[C,U]                         |

## b) ANALYSIS OF PANCREATIC RNase PRODUCTS FROM THE 30-NUCLEOTIDE FRAGMENT

| Pancreatic RNase primary product | Products of secondary digestion with T <sub>1</sub> RNase |                         |                         |                         | Sequence deduced         |
|----------------------------------|-----------------------------------------------------------|-------------------------|-------------------------|-------------------------|--------------------------|
|                                  | [α- <sup>32</sup> P]CTP                                   | [α- <sup>32</sup> P]UTP | [α- <sup>32</sup> P]GTP | [α- <sup>32</sup> P]ATP |                          |
| m30/P1                           | C                                                         | C                       | C                       | —                       | GGC[U]                   |
| m30/P2                           | U                                                         | C                       | C                       | —                       | GGU[C]                   |
| m30/P3                           | AC                                                        | —                       | —                       | AC                      | AC[A]                    |
| m30/P4                           | C                                                         | —                       | C                       | C                       | GC[A,G]                  |
| m30/P5                           | —                                                         | AC                      | AG                      | —                       | AG[U]                    |
| m30/P6                           | AU                                                        | AAU                     | AU                      | —                       | AU[C,U,G]                |
| m30/P7                           | cap                                                       | cap                     | cap                     | —                       | m7Gppp <sup>m</sup> C[U] |
| m30/P8                           | —                                                         | —                       | C                       | C                       | C[G,A]                   |
| m30/P9                           | U                                                         | U                       | —                       | U                       | U[C,U,A]                 |

Fig. 4 Analysis of T<sub>1</sub> and pancreatic RNase products from fragment m30. See Fig. 3 legend for explanations.

sequence G<sup>m</sup>CUA adjacent to the cap is not seen, however, in many other messages, including brome mosaic virus RNA 4 (m7GpppGUAU)<sup>3</sup>, vesicular stomatitis virus mRNA (m7GpppA<sup>m</sup>AC)<sup>16</sup>, silk fibroin (m7GpppA<sup>m</sup>U<sup>m</sup>C)<sup>17</sup> and rabbit globin (m7Gpppm<sup>6</sup>A<sup>m</sup>C<sup>m</sup>AC)<sup>18</sup>. The sequence AUGG, common to all six reovirus messages as well as globin mRNA<sup>4,5</sup>, has the striking potential to form four base pairs with the anticodon loop (CCAU) of mammalian initiator tRNA<sup>22</sup>. In brome mosaic virus RNA 4, however, the AUG initiation codon is not followed by a G residue<sup>3</sup>. Thus, homology among the eukaryotic regions so far sequenced is limited to presence of an AUG triplet and a 5'-terminal cap.

It is difficult to detect any other significant features common to eukaryotic initiation sites. For example, eukaryotic ribosome binding regions differ from one another in that the AUG triplet does not always occur in the same phase, determined by measuring off three nucleotides at a time, from the AUG leftwards to the cap. The distance between the cap and the AUG also varies, from 11 nucleotides<sup>3</sup> to at least 55 nucleotides<sup>4,5</sup>. Furthermore, nonsense codons, which commonly occur on the 5' side of the initiation triplet in prokaryotic ribosome binding sites<sup>11</sup>, are not a universal feature of eukaryotic initiation sites, since reovirus fragments s54, s46 and m30 lack such codons. The suggestion has been made that a specific interaction, involving a region near the AUG codon which is complementary to the 3' end of 18S ribosomal RNA, might be characteristic of eukaryotic (as well as prokaryotic) initiation sites<sup>2</sup>. Between the reovirus 5'-terminal fragments and the 3'-terminal eight residues of 18S rRNA<sup>19</sup>, however, there are only minimal opportunities for base pairing (involving only two base pairs in fragments s54, s46 and m30; three base pairs in s45 and m44; and four A-U base pairs adjacent to the cap in fragment m52). Although absence of significant complementarity cannot be proved until additional portions of the 18S rRNA sequence

become known, there is no evidence in eukaryotic systems for even a moderately-stable, specific interaction between messenger and 18S ribosomal RNA. This seems to be true not only for reovirus, but also for the 5' non-coding regions of rabbit globin<sup>4,5</sup>, brome mosaic virus<sup>3</sup> and SV40 VPI messages<sup>20</sup>. Although base pairing with ribosomal RNA has been postulated for those three messages, the proposed interactions (involving primarily A-U base pairs, interrupted by unpaired residues in the case of SV40 VPI) are predicted to be far less stable than those demonstrated with prokaryotic messages<sup>2</sup>. Furthermore, while the region postulated to undergo base pairing with ribosomal RNA is located in a rather constant position relative to the AUG in prokaryotic messages, this is not true of the eukaryotic initiation sites<sup>5</sup>. Thus, the suggestion that base pairing with ribosomal RNA might help to position the ribosome on eukaryotic messages seems to derive more from analogy with prokaryotic systems than from predication based on the available eukaryotic ribosome binding sequences. Although eukaryotic cellular mechanisms often parallel those observed with prokaryotes, perhaps the process of translational initiation is exceptional, due to the structural differences between prokaryotic and eukaryotic messages. With polycistronic prokaryotic messages, which require discrimination between the few internal AUG and GUG codons that actually serve for initiation and the much larger set of internal AUG and GUG codons which normally do not function as initiation triplets, a relatively intricate mechanism may be required for selection of the correct initiation sites by ribosomes. With monocistronic eukaryotic messages, on the other hand, initiation requires recognition of a single AUG codon, and inspection of the available eukaryotic sequences suggests that initiation begins at the AUG nearest the 5' end of the message. (Although the SV40 VPI sequence<sup>20</sup> seems to be an exception, the possible occurrence of very limited processing of the VPI protein, involving removal of a few N-terminal amino acids, would hamper the correct identification of the initiation region, which was based not on ribosome binding but on a comparison of the nucleotide sequence of the DNA with the amino acid sequence of the mature virion protein.) Inclusion of the 5' terminus as well as the AUG triplet within all six 40S ribosome-protected reovirus fragments is consistent with the possibility that these two elements are necessary features (recognition signals) in eukaryotic initiation sites. The absence of other detectable homologies among the limited set of eukaryotic initiation regions sequenced to date may mean that the presence of an AUG triplet near a 5' terminus (usually, but not necessarily capped) is sufficient to direct binding of eukaryotic ribosomes. This 'minimal recognition' hypothesis will undoubtedly require modification as more data becomes available. For example, if an AUG codon in the 5' region of an RNA molecule were masked by the surrounding secondary structure, that AUG might not mediate initiation unless a mechanism were available to denature the RNA. Furthermore, an AUG codon located extremely close to the 5' terminus, as in Sindbis 42S virion RNA (m7GpppAUG)<sup>21</sup>, would probably be non-functional because of steric considerations. We suggest, based on evidence to be published elsewhere, that the methylated cap functions secondarily, in a way that does not affect where the ribosome attaches to the message but that greatly increases the yield of initiation complexes. The secondary and tertiary structure of the intervening region, between the 5' terminus and the AUG triplet, might also be expected to influence translational efficiency by determining the spatial orientation of the two putative recognition elements. Thus, although the presence of an AUG codon near the 5' end of an RNA chain might be sufficient to permit a certain low level of ribosome binding, other structural features in the RNA probably modulate the initiation event.

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# letters to nature

## Identification of cosmic $\gamma$ -ray sources CG135+1 and CG189+1 with HII regions

ELEVEN unidentified  $\gamma$ -ray sources have been reported by the COS B group<sup>1</sup> one of which was previously known from the SAS 2 satellite<sup>2</sup>. Here, I point out two likely associations of the new sources with giant HII regions, OB associations, molecular clouds and dust in the Cas OB6 region of the Perseus arm. An Ha photograph and identification chart for this region is given in ref. 3. The HII region W3 (IC1795) is located at ( $l = 133.7^\circ$ ,  $b = +1.1^\circ$ ), which is only  $0.7^\circ$  outside the one standard deviation error box for CG135+1 ( $l = 135.5^\circ \pm 1.0^\circ$ ,  $b = 1.5^\circ \pm 1.0^\circ$ )<sup>1</sup>. The SAS 2 longitude distribution<sup>4</sup> is consistent with the existence of this source.

W3 lies at a distance of  $\sim 3.1$  kpc in the Perseus arm<sup>5</sup>, and contains 10 infrared sources<sup>6,7</sup>, four condensed centimetre sources W3(A,B,C,D) (ref. 8) and an OH maser source<sup>8</sup>. A spectrum of W3(A) from 408 MHz– $10^{14}$  Hz is given in ref. 9. Krügel and Mezger<sup>10</sup> suggest that W3 is an O-star association in a very early evolutionary stage, the component W3(A) consisting of a compact dense shell of ionised gas surrounded by a dense shell of neutral gas (density  $\sim 10^4$  cm<sup>-3</sup>). The total infrared luminosity is  $\sim 10^6 L_\odot$  (ref. 11); 1-mm observations<sup>12</sup> suggest a column density for W3 of  $10^{23}$  H<sub>2</sub> molecules cm<sup>-2</sup>, and an H<sub>2</sub> mass of  $10^3 M_\odot$ .

The reported intensity of CG135+1 is  $\sim 2 \times 10^{-6}$  photons ( $> 70$  MeV) cm<sup>-2</sup> s<sup>-1</sup> (ref. 13). If the cosmic-ray proton intensity is the same in W3 as locally, we can estimate the intensity of  $\pi^0$ -decay  $\gamma$  rays using the source function given by Stecker<sup>14</sup> to be  $\sim 1.6 \times 10^{-10}$  photons ( $> 70$  MeV) cm<sup>-2</sup> s<sup>-1</sup>. Bremsstrahlung interactions may increase this by up to a factor  $\sim 2$  (ref. 15) if the  $e/p$  ratio is the same in the cosmic-radiation flux near W3 as locally. Thus if the identification is correct, a density of cosmic radiation  $\sim 10^4 \times$  the local density is required; this might be plausible if the sources of cosmic rays were associated with the young stars in the HII regions. The particles could either have been accelerated in the stars themselves, or in an associated supernova or SNR (the SNR HB3 is nearby and may be physically connected with W3<sup>16,17</sup>). The association of W3 with the  $\gamma$ -ray source, however, could be attributed to an unseen  $\gamma$ -ray pulsar (there is no known radio pulsar in this direction) or an unknown  $\gamma$ -ray emitter connected with young stellar populations.

I also suggest the identification of the source CG189+1 ( $l = 189.0^\circ \pm 1.5^\circ$ ,  $b = 1.0^\circ \pm 1.5^\circ$ )<sup>1</sup> with the HII region NGC2175 at  $l = 190^\circ$ ,  $b = \pm 0.5^\circ$ . Aperture synthesis observations of this object, and a report of an associated CO cloud 20 pc in diameter, have been published<sup>18</sup>.

NGC2175 has the same 6-cm flux density (30 Jy) as W3(A)<sup>19</sup> and should have a similar infrared intensity to W3(A) if the proportionality of infrared to centimetre continuum applies<sup>11</sup>. The positional coincidence here is in fact better than for W3.

The  $\gamma$ -ray source is extended in latitude<sup>1</sup>, and also includes the direction to the SNR IC 443 ( $l = 189^\circ$ ,  $b = -3.2^\circ$ )<sup>20</sup> which is an X-ray source<sup>21</sup>. The distance to both IC 443 and NGC 1275 is about 1.5 kpc, so that the possibility of a physical connection is not ruled out, in which case the situation may provide a case similar to that for W3 and HB3 discussed above.

If  $\gamma$ -ray emission is a common feature of objects like W3, other objects with similar radio and infrared fluxes should be  $\gamma$ -ray sources. Of the objects listed in refs 7 and 11, only W75 lies within the four areas examined by the COS B group<sup>1</sup>. W75 ( $l = 81.9^\circ$ ,  $b = 0.8^\circ$ ) lies in the very densely populated Cygnus region in which the visibility of individual sources is rather low. The nearest  $\gamma$ -ray source is CG78.1 ( $l = 78.5^\circ \pm 0.5^\circ$ ,  $b = 1.5^\circ \pm 0.5^\circ$ )<sup>1</sup>.

The Orion complex (M42) should be easily observable if the W3 identification is correct, since it is about five times as intense both at 30–300  $\mu$ m and at 2 cm (ref. 11), and is in a region away from the Galactic plane where point-source visibility should be good. But it lies outside the four regions examined by Hermesen *et al.*<sup>1</sup>, M17 is also a good candidate, being brighter than W3 in both infrared and radio; however, it is in the galactic plane at longitude  $14.7^\circ$ , and hence harder to distinguish from the galactic background.

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## Tidal interactions and the massive halo hypothesis

THE idea that galaxies may be surrounded by large amounts of unseen material has become very popular. The arguments favouring such massive haloes are almost all based on the dynamical analysis of galaxies either individually or in groups, and they have been cogently assembled by Einasto *et al.*<sup>1</sup> and by Ostriker *et al.*<sup>2</sup>. They concluded that galaxies may well be surrounded by large ( $\sim 100$  kpc) or very large ( $\sim 0.5$  Mpc) 'isothermal haloes' whose mass varies as the radius within which it is measured. (We take  $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ ). Their views have subsequently received support from Arecibo observations which found flat HI rotation curves to very large radii for several spiral galaxies<sup>3</sup>, and from analyses of the dynamics of new samples of binary galaxies<sup>4</sup> and of groups of galaxies<sup>5</sup>. The most extreme form of the massive halo hypothesis postulates very large haloes which are the dominant constituent of the Universe, and in which the observed galaxy is little more than contaminating debris left over from the formation of the unseen object<sup>6</sup>. It is disquieting that there seems to be little chance of direct observation of this dark material, and this letter points out that dynamical constraints can be put on either the extent or the universality of galactic haloes.

When a cluster of galaxies collapses and comes to equilibrium, the structure of the subunits which were its progenitors is rapidly broken up by the violent relaxation of the cluster as a whole<sup>7</sup>; similarly, if the individual galaxies in a group are too large, they may interact strongly enough that the whole group is soon smoothed into a single unit. Richstone showed that even if the galaxies in the core of the Coma cluster had once had sufficiently large haloes to contain the missing mass, their mutual interactions would by now have consigned 90% of the material to an intergalactic pool<sup>8</sup>. Galaxy-galaxy interactions are weakened by high encounter velocities in Coma, however, and should be much stronger in small groups where the velocity dispersion is of the same order as the internal dispersion of the individual galaxies: if the tidal destruction of galaxy-halo units has gone most of the way to completion in Coma, it may have gone entirely to completion in many groups.

The consequences of tidal interactions for the massive halo hypothesis are most easily discussed for binary galaxies. If haloes of almost unlimited extent form around most galaxies, the mass distributions of the members of a binary will overlap, and they may well orbit within a common envelope. Tidal effects will then cause the loss of orbital energy into internal energy and the merging of the observed galaxies. S.D.M.W. has recently carried out full N-body simulations of the interaction between pairs of 250-particle 'galaxies', and has found that if two equilibrium 'galaxies' are placed in a bound orbit in which their mass distributions significantly overlap, they merge very rapidly (compare ref. 9). The details of these calculations will be published elsewhere, but this statement may be quantified by noting that, for the near isothermal ( $\rho \sim r^{-2}$ ) 'galaxies' studied, the condition  $r_{\text{peri}} \lesssim 3r_i$  was sufficient for the binary to merge completely within one initial orbital time of its first close approach, where  $r_{\text{peri}}$  is the pericentric distance of the initial orbit and  $r_i$  is the half-mass radius of the initial 'galaxies'.

The dire implications of this result for the hypothesis of universal massive haloes can be seen from Fig. 1 which plots the quantity  $N = \Delta v / (2)^{1/2} \pi H_0 r_p$  against magnitude difference for the 59 statistically selected binaries in Turner's sample<sup>4</sup> ( $\Delta v$  and  $r_p$  are the observed velocity difference and projected separation); note that the galaxies in most of the pairs are of comparable magnitude. On the assumption of similar randomly-oriented circular orbits the median value of  $N$  is an unbiased estimator of the number of orbits the binaries have executed in a Hubble time (taken as  $H_0^{-1}$ ); it should be close to the median number of actual orbits for the observed sample. The median pair has apparently execu-

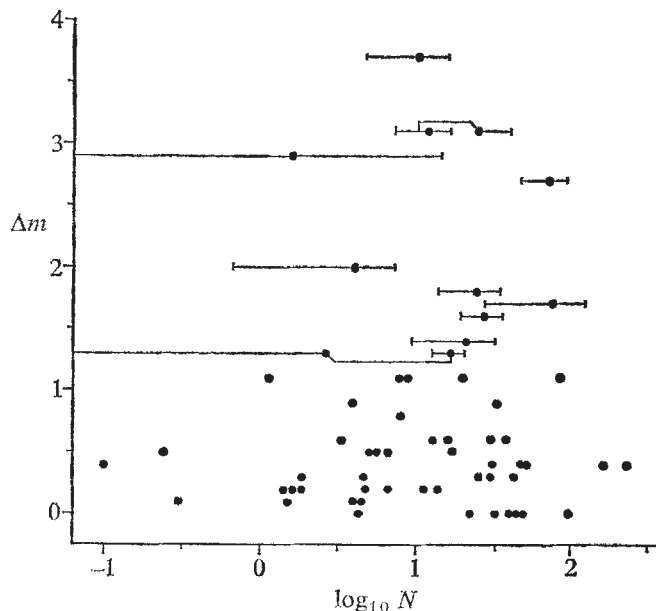


Fig. 1 The  $\Delta m$ - $\log N$  distribution for the 59 binaries in Turner's sample.  $\Delta m$  is the magnitude difference between the pair members and  $N$  is the estimated number of orbits completed in a Hubble time. Errors in  $\log N$  have been calculated for representative points using Turner's quoted uncertainties in  $\Delta v$  and  $r_p$ .

ted 13 revolutions since the beginning of the Universe: either Turner's binaries do not overlap substantially anywhere in their orbits, or they will spiral together in the next few billion years. Discounting optical pairs there are about 139 true binaries in the Zwicky catalogue satisfying all Turner's selection criteria; if their median remaining lifetime is equal to their median apparent orbital time, then roughly  $13 \times \frac{1}{2} \times 139 \approx 900$  similar pairs should have merged in the past. This number is 20% of all the galaxies in the correct magnitude range in the part of the sky surveyed; it is likely to be a severe underestimate of the total number of binaries which have merged in the past both because of the stringency of the sample selection criteria and because the initial distribution of separations may have been more strongly peaked at small radii than the distribution implicitly assumed above. We estimate that these effects could increase the percentage of merged binaries by a factor between two and five.

If one is unwilling to accept that most of Turner's pairs will merge during their next orbital period, one can ask how small their haloes need to be to avoid strong tidal interactions. The mean projected separation of the 59 pairs in the sample is 79 kpc. Under the assumption that the distribution of pair separations is a power law and is independent of the magnitudes of the constituent galaxies, the correction factor from mean projected separation to mean true separation is easily calculated. For all pairs with given magnitudes at a given distance,

$$\frac{\bar{r}_p}{r} = \frac{\int_0^\infty dr \int_0^{\pi/2} d\theta \sin\theta f(r \sin\theta, D) n(r) r \sin\theta}{\int_0^\infty dr \int_0^{\pi/2} d\theta \sin\theta f(r \sin\theta, D) n(r) r}$$

where  $\theta$  is the angle between the separation vector and the line of sight,  $n(r)$  is the distribution of separations, and  $f(r \sin\theta, D)$  is the probability of inclusion as a function of projected separation at distance  $D$ . If  $n(r) \propto r^{-\gamma}$  each integral can be separated into

an integral over  $r \sin \theta$  which cancels out of the fraction, and an integral over  $\theta$  which remains to give

$$\frac{\bar{r}_p}{\bar{r}} = \int_0^{\pi/2} d\theta \sin^{\gamma} \theta / \int_0^{\pi/2} d\theta \sin^{\gamma-1} \theta \quad (1)$$

This result can be expressed in terms of  $\Gamma$ -functions for all  $\gamma > 0$ . Since equation (1) does not involve  $D$  it holds for the sample as a whole, and the correction factor is independent of whether the averages are number or luminosity weighted. Turner obtains  $\gamma = \frac{1}{2}$  for his sample, giving  $\bar{r}_p/\bar{r} = 0.46$ ; the mean true separation of his pairs is, therefore, 173 kpc, and if they are not to be strongly interacting we require  $r_i < 58$  kpc on average for the haloes of the constituent galaxies. This limit must be reduced if the orbits are sensibly eccentric since the condition for merging applies to the distance of closest approach, whereas most of the pairs will be seen near apocentre.

Although the above arguments are crude, they demonstrate that while certain dynamical considerations seem to require massive haloes, there are others which can severely constrain them.

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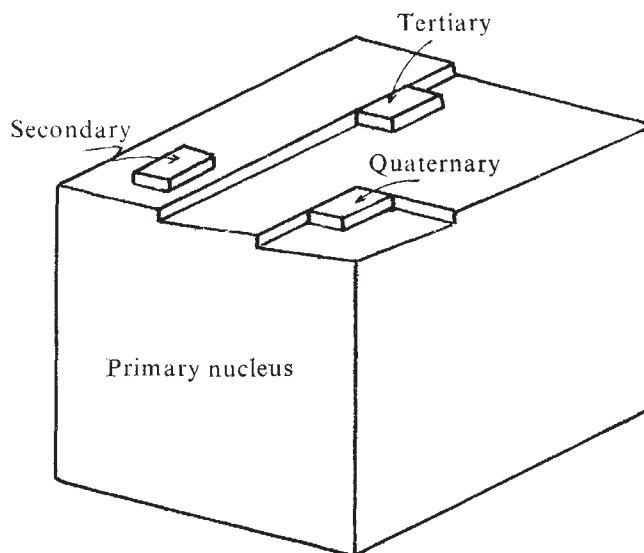
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## Surface area and secondary nucleation theory

PRIMARY nucleation theory involves estimating the maximum free energy necessary to form a crystal of sufficient size so that further crystallisation results in a decrease in free energy. This free energy increase is caused by the creation of surfaces with the liquid or vapour. (The number of sides will depend on crystal symmetry and crystal habit. Here we will consider only the simplest six-sided crystal.) After a crystal of this minimum size has been formed it is often necessary to consider a secondary nucleation process which involves the maximum free energy necessary to crystallise a new layer on one of the existing flat crystal surfaces. This note reports an error in the estimation of surface area in the theory of secondary nucleation processes. This error makes little difference when applied to the crystallisation of small molecules but it can have significant results in the crystallisation of polymer molecules.

The secondary nucleation process controls the growth rate even after the primary crystal has grown macroscopically large. Again the cause of this free energy increase is assumed to be the creation of new surfaces between the crystal and the liquid (or vapour). It has become traditional in such nucleation processes to count only four of the five surfaces which such a secondary nucleus has exposed to the liquid. The argument being that the fifth surface simply replaces an equivalent surface on the parent crystal and



**Fig. 1** Illustration of the four nucleation processes. Primary nucleation controls the number of crystals. Secondary nucleation controls the crystal growth rate. In polymer crystallisation tertiary nucleation controls the molecular fractionation process and quaternary nucleation may influence the morphology. In all of these processes the free energy of the nucleus is influenced not only by the surface energies but also by the change in conformational entropy of any attached cilia.

thus does not contribute to the increase in the total free energy. But the parent crystal may now be so large that any increase in the surface area of a secondary nucleus is insignificant in relation to the total free energy. The total free energy change reaches its maximum value at the critical size for the primary nucleus. It is not the change in total free energy which is important in secondary nucleation but rather the change in the free energy of the secondary nucleus considered as a small thermodynamic system<sup>1</sup> which controls secondary nucleation. A small thermodynamic system is one whose extensive properties (such as its free energy) may not be considered to be precise linear homogeneous functions of the thermodynamic variables. In such a system the surfaces have a dominant role in determining the free energy and one must consider all the surfaces which the small thermodynamic system makes with its surroundings. A coherent 'surface' which a secondary nucleus makes with the parent crystal may be considered to have zero surface energy but all surfaces which the nucleus makes with the liquid (or vapour) will contribute to the thermodynamic properties of the nucleus.

This distinction is not important when the crystallising entity is an atom or small molecule, since the secondary nucleus will always be one molecule in thickness, (or even if it is not, no difference in morphology can be distinguished) and the values of the surface energy derived from the kinetics of crystallisation have little intrinsic use except for comparison with other similarly derived data. But in considering the crystallisation of polymer molecules, the neglect of this fifth surface has led to the conclusion that such molecules should also crystallise in a monolayer of regularly folded chains<sup>2–4</sup>.

**Table 1** Number of crystal faces (six-sided crystal)

|                       | Classical | Small system |
|-----------------------|-----------|--------------|
| Primary nucleation    | 6         | 6            |
| Secondary nucleation  | 4         | 5            |
| Tertiary nucleation   | 2         | 4            |
| Quaternary nucleation | 0         | 3            |

Although this assumption has led to reasonable results<sup>5</sup> in explaining crystal growth kinetics and chain fold lengths, there are some experimental results which are incompatible. For example, large extended chain polyethylene crystals with very obvious striations parallel to the chain direction which should provide ideal secondary nuclei have been shown<sup>6</sup> to be ineffective in promoting crystal growth when exposed to molten polyethylene substantially below its melting temperature. Furthermore, when the temperature is lowered sufficiently to allow crystallisation to proceed it does so by chain folding rather than extending along the existing secondary nucleation sites. These results can be explained by including all surfaces which a crystallising molecule makes with the liquid in the estimation of its free energy change<sup>7</sup>. Table 1 summarises the number of surfaces for each type of nucleation for both the classical and the small system approach.

Since tertiary nucleation and quaternary nucleation produce no detectable changes in atomic or small molecular crystallisation they need only be considered in polymer crystallisation where they are important in controlling morphology and molecular fractionation. In addition, in polymer crystallisation one may have surfaces exposed to the liquid which have attached portions of uncrystallised molecules. These attached molecules (sometimes called cilia) make important differences in the conformational entropy change and when added to the surface energies of the various nucleation processes make up the molecular nucleation process suggested by Wunderlich<sup>8</sup>.

The principal difference in applying this small system thermodynamic approach is the conclusion that secondary nucleation of polymer molecules need not result in a monolayer of regularly folded chain crystals but more likely will produce a multi-layer nucleus in which each molecule is folded into a more compact bundle with a random arrangement of folds.

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## Crystal structure of alinite

ALITE, a major constituent<sup>1</sup> of Portland cement, is essentially tricalcium silicate  $\text{Ca}_3(\text{SiO}_4)\text{O}$ , which contains both  $(\text{SiO}_4)^{4-}$  and free  $\text{O}^{2-}$  ions<sup>2,3</sup>. It is of interest to cement technology to examine how chloride contamination of the raw materials affects the phases present in the resulting cement, and the extent to which the free oxide ions of tricalcium silicate are replaceable by  $\text{Cl}^-$ . While this replacement can be regarded as complete in the compound<sup>4,5</sup>  $\text{Ca}_3\text{SiO}_4\text{Cl}_3$ , it is only partially accomplished in the new compound alinite, which is described in this note.

Single crystals of the new compound alinite were obtained from cement clinker containing additional Mg, Al and Cl. The crystals have diffraction symmetry  $I4/mmm$ , with  $a = 10.4714$  and  $c = 8.6171$  (16) Å. In the completed structure, in the space group  $I42m$  (no. 121), with  $R = 0.034$ , based on 401 independent reflections, the unit cell contains two structural units of ideal formula  $\text{Ca}_{11}(\text{Si}_{0.75}, \text{Al}_{0.25})_4\text{O}_{18}\text{Cl}$ . The structure exhibits

Table 1 Alinite—atomic parameters

| Atom | No. per cell | Fractional coords ( $\times 10^4$ ) |         |         | $B_{\text{iso}}$<br>(Å <sup>-1</sup> $\times 10^3$ ) | Occupancy |
|------|--------------|-------------------------------------|---------|---------|------------------------------------------------------|-----------|
|      |              | $x/a$                               | $y/b$   | $z/c$   |                                                      |           |
| Ca1  | 16           | -6(4)                               | 2609(1) | 2914(1) | 96                                                   | 1.09      |
| Ca2  | 2            | 0                                   | 0       | 0       | 39                                                   | 1.12      |
| Ca3  | 4            | 0                                   | 1/2     | 0       | 92                                                   | 0.96      |
| Si   | 8            | 2107(1)                             | 2107(1) | 5(6)    | 32                                                   | 1.11      |
| O1   | 16           | 1782(3)                             | 3652(3) | -101(9) | 88                                                   | 1.10      |
| O2   | 8            | 1439(4)                             | 1439(4) | 1561(5) | 48                                                   | 1.10      |
| O3   | 8            | 1450(5)                             | 1450(5) | 8489(6) | 81                                                   | 1.11      |
| O4   | 4            | 0                                   | 1/2     | 1/4     | 82                                                   | 1.09      |
| Cl   | 2            | 0                                   | 0       | 1/2     | 168                                                  | 1.05      |

Estimated standard deviations are given in parentheses.

isolated alumino-silicate tetrahedra within a framework consisting of three distinct types of Ca coordination polyhedra.

Intensity data ( $\sin^2\theta \leq 0.28$ ) was obtained with an Enraf-Nonius CAD-4 diffractometer and  $\text{MoK}\alpha$  radiation. Solution of the three-dimensional Patterson (vector) map by multiple superposition (the rhombus method<sup>6</sup>) yielded positions for five atoms, one of which was taken to be Si and assigned the  $f$ -curve for  $\text{Si}^{4+}$ , while the remainder were treated as  $\text{Ca}^{2+}$ . Three cycles of structure factor calculation (in  $I4/mmm$ ) followed by the preparation of electron density maps not only revealed the positions of all of the remaining (oxygen) atoms, but also identified the

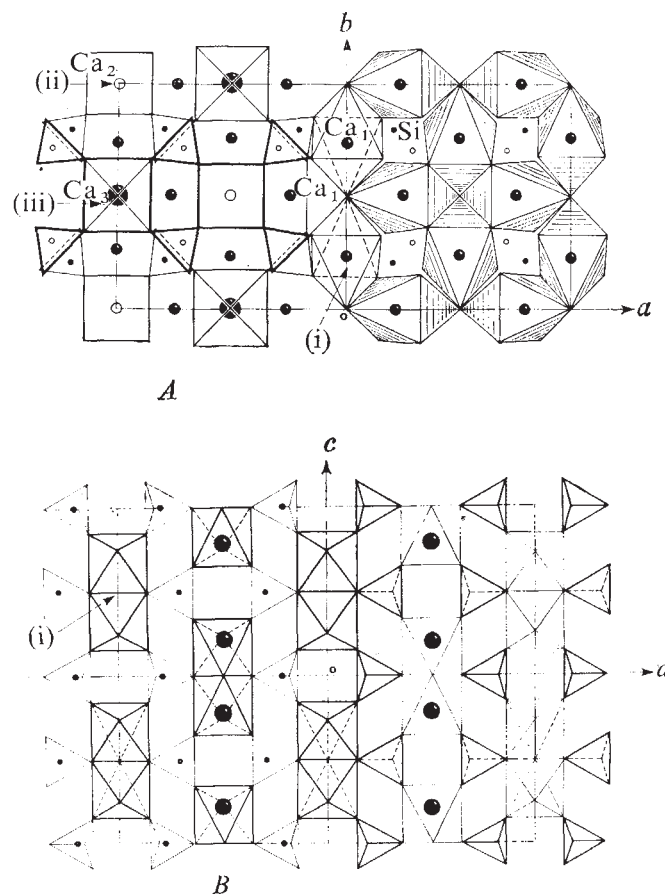


Fig. 1 The structure of alinite—general features. For clarity, representative Cl and O positions are indicated, but only as vertices of polyhedra. A, (001) projection, showing the three types of Ca coordination polyhedra and (Si, Al) tetrahedra. The smallest circles are (Si, Al) positions—● and ○ indicate  $z \approx 0$  and  $z \approx 0.5$  respectively. Intermediate ○ are Ca2. Intermediate ● represent overlapping pairs of Ca1 atoms ( $z \approx 0.29$  and  $0.71$  at the cell edges;  $z \approx 0.79$  and  $0.21$  elsewhere). The largest ● represent overlapping pairs of Ca3 atoms ( $z = 0$  and  $0.5$ ). B, One layer of the structure parallel to (010) at  $y = \frac{1}{2}$  showing pairs of face-sharing Ca1 octahedra and (Si, Al) tetrahedra—large and small circles represent Ca1 and (Si, Al) atoms respectively.



original atoms as 3 Ca, 1 Si and 1 Cl, completely independently of any analytical data. Block diagonal least squares refinement of atomic positions (throughout) with alternate refinement of occupancies and  $B_{iso}$  led to  $R = 0.09$ . The space group  $I\bar{4}2m$ , however, at a similar stage of refinement gave  $R = 0.08$ , and finally after refinement of anisotropic temperature factors  $R = 0.034$ . The final parameters are given in Table 1.

Although the completed structure indicates the contents of the unit-cell as  $Ca_{11}Si_4O_{18}Cl$ , charge balance requires partial occupancy of the Si position by Al ( $Si_{0.75}, Al_{0.25}$ ), to give the overall composition given above. The conditions in which the phase is obtained, and preliminary analytical data support this contention.

The most frequently occurring Ca coordination is that of Cal. For example, indicated by i in Fig. 1A, Cal (at 0.0, 0.26, 0.29) has as nearest neighbours: Cl(0.0,  $\frac{1}{2}$ ); O1(0.13, 0.32, 0.49); O1' (-0.13, 0.32, 0.51); O2(0.14, 0.14, 0.16); O3(-0.15, 0.15, 0.15) and O4 (0,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ). Each such octahedron shares its Cl-O1-O1' face with a second similar octahedron, related to the first by a two-fold axis passing through the Cl atom and the mid-point of the common O1-O1' edge ((i)—Fig. 1B). Such pairs of Cal octahedra occur in four-fold clusters (eight octahedra in all) round the 0,0,z lattice row, sharing Cl-O2 and Cl-O3 edges. The O2 and O3 vertices of two such clusters provide the cubic coordination of Ca2 ((ii)—Fig. 1A). The Cal octahedron pair units are also further associated in pairs (four octahedra in all) centred on the 0,  $\frac{1}{2}$ , z lattice rows. In this case the O4 (0,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ) and O4' (0,  $\frac{1}{2}$ ,  $\frac{3}{4}$ ) common to both Cal octahedron pairs, together with the two shared O1-O1' edges (one from each pair) complete the octahedral coordination of Ca3 (0,  $\frac{1}{2}$ ,  $\frac{1}{2}$ ) ((iii)—Fig. 1A). A similar arrangement of four Cal octahedra, but with the octahedron pair interaction at right angles to that given immediately above applies to Ca3 at 0,  $\frac{1}{2}$ , 0. Figure 1A and B also indicates the distribution of the aluminosilicate tetrahedra.

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## The nature of metastable phases of carbon

THE following forms of carbon are already known: rhombohedral and hexagonal graphite, diamond, lonsdallite, carbines, Ries crater carbon, and metallic carbon. We describe here new metastable phases in the carbon system<sup>1,2</sup> which we detected while sintering highly dispersed diamond powders. Similar structures were also observed during explosive transformations of pyrographite<sup>3</sup> and in natural diamonds<sup>4</sup> that had undergone shock metamorphism. In all cases, the observed structures are close to those of the Ries crater carbon<sup>5</sup>.

Under pressures > 600 kbar, carbon passes into the metal phase (C3)<sup>6,7</sup>. When sintering diamond powders, it may be shown that the pressures set up between particles within the

contact interaction zone, substantially exceed the mean hydrostatic pressures.

By using the Hertz solution on the interaction of two balls<sup>8</sup>, it is possible to find the radius of the zone of contact of two balls<sup>8</sup> (that approximate the diamond grains) of radius  $R$

$$a = \left( \mathcal{F}_1 \frac{\mathcal{D}R}{2} \right)^{1/3} \quad (1)$$

where

$$\mathcal{D} = \frac{3}{2} \left( \frac{1 - \sigma^2}{E} \right)$$

Here  $\mathcal{F}_1$  is the mean force applied to one sphere,  $\sigma$  is the Poisson coefficient,  $E$  is the Young modulus. The mean pressure set up on the area does not depend on the particle radius and is equal to

$$P = P_0^{1/3} \left( \frac{2}{\pi \mathcal{D}} \right)^{2/3} \quad (2)$$

where  $P_0$  is the mean pressure applied to the chamber in sintering. In the case of diamond  $\mathcal{D} \approx 1.5 \times 10^{-7} \text{ bar}^{-1}$ , if we assume  $E = 9.5 \cdot 10^{12} \text{ dyn cm}^{-2}$ ,  $\sigma = 0.25$  (ref. 9). Then,

$$P = 2.7 \times 10^4 P_0^{1/3} \quad (3)$$

Thus, if the pressure is applied such that  $P_0 = 30 \text{ kbar}$ , the mean pressure within the contact zone will exceed 800 kbar. Therefore, when sintering the diamond powders, the appearance

**Table 1** Calculation of interplanar spacing ( $d$ ) of the sintered diamond powder

| Starting<br>$d(\text{\AA})$ | 600 °C<br>$d(\text{\AA})$ | 800 °C<br>$d(\text{\AA})$ | Notes       |
|-----------------------------|---------------------------|---------------------------|-------------|
| 3.326                       | 3.43                      | 3.338                     | Graphite    |
| 3.010                       | 3.06                      | —                         | Ries carbon |
| —                           | 2.29                      | —                         | Ries carbon |
| —                           | 2.12                      | —                         | Graphite    |
| 2.045                       | 2.07                      | 2.062                     | Diamond     |
| 1.965                       | —                         | —                         | Lonsdallite |
| —                           | 1.52                      | —                         | Lonsdallite |
| 1.291                       | —                         | —                         | Ries carbon |
| 1.258                       | 1.27                      | 1.27                      | Diamond     |
| 1.234                       | —                         | 1.23                      | Graphite    |
| 1.074                       | 1.08                      | 1.078                     | Diamond     |
| 0.900                       | 0.894                     | 0.894                     | Diamond     |
| 0.818                       | 0.818                     | 0.820                     | Diamond     |

of metallic carbon may be expected. As the metallic carbon may prove to be a metastable phase, it can pass into other forms of carbon. The additional phases that are observed in sintering diamond powders may turn out to be intermediate states between the metallic carbon, on one side, and the diamond and graphite, on the other. With this in mind, an experiment was carried out, in which the diamond powder having the mean radius of particles of about 2  $\mu\text{m}$  was subjected to compression at the mean pressure of 59 kbar and the temperature of 1,200 °C. Then the diamond powder was annealed in a hydrogen medium at temperatures of 600 °C and 800 °C for 1 h. The initial and the annealed powder were subjected to X-ray examination according to the Debye method. The examination results are presented in Table 1.

From Table 1, it seems that many interplanar spacings ( $d$ ) are recorded in the initial powder, this may be attributed to different forms of carbon. In annealing, the system passes to a state where only diamond and graphite are present.

**Table 2** Results from the pulse-heated diamond powder

| Intensity<br>(relative units) | <i>d</i><br>(Å) | <i>d</i> diamond<br>(Å) | Note              |
|-------------------------------|-----------------|-------------------------|-------------------|
| 100                           | 2.05            | 2.06                    |                   |
| 10                            | 1.746           |                         | β-carbide (1.750) |
| 5                             | 1.523           |                         | β-carbide (1.550) |
| 80                            | 1.260           | 1.261                   |                   |
| 70                            | 1.073           | 1.075                   |                   |
| 60                            | 0.891           | 0.892                   |                   |
| 70                            | 0.818           | 0.818                   |                   |

In the case of the pulse radiant heating of the diamond powder up to 2,000 °C and its rapid cooling (which obviates the annealing of metastable states), additional phases also appear, as shown by Table 2.

From Table 2, it seems that the carbides should occupy the intermediate place between diamond and graphite.

One possibility is that, aside from the known crystallographic forms in the carbon system (like the boron nitride system), there exist transient metastable states whose stability is determined by the potential barrier height. Transition from one form of carbon to another may occur, either via those metastable states or, possibly, directly.

The following seems probable: the Ries crater carbon, which has formed on the place where a gigantic meteorite has fallen, is a product of the inverse transition of metallic carbon (or the phases that are close to it) into a state which is more stable under atmospheric pressure.

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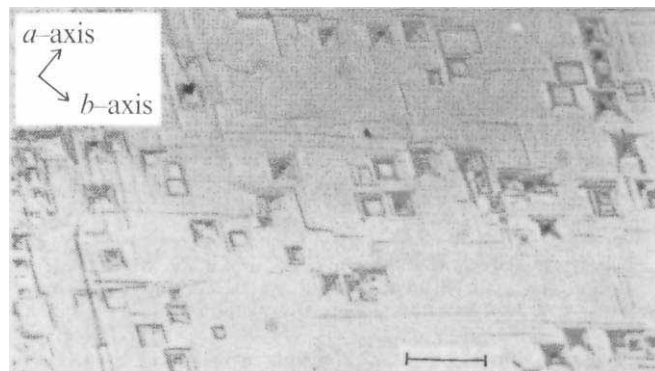
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## Etching on large single crystals of stearic acid

ETCHING is one of the most useful ways of directly observing dislocations in a crystal<sup>1</sup>. Many studies have been made of dislocations in organic crystals, such as aromatic hydrocarbons<sup>2</sup>, but no reports on the dislocations in fatty acid crystals have been published because no large single crystal could be obtained. Recently we have succeeded in obtaining large single crystals of the saturated fatty acid stearic acid from solutions of organic solvents<sup>3</sup>. We describe here attempts to etch single stearic acid crystals using organic solvents. Optical and transmission electron micrographs of etch pits reveal several novel features and give an apparent correlation between the pits and the dislocations in stearic acid crystals.

Crystals of stearic acid were grown from a solution in benzene; the average size was about 5 × 5 mm<sup>2</sup> in area and 1 mm thick. The crystal form was monoclinic (B-form)<sup>3</sup>. The long chain of the stearic acid molecule was directed towards the (001) direction.

Etch pits were optically observed on the as-grown (001), *ab*, surfaces (Fig. 1). Attempts to etch other crystal surfaces were



**Fig. 1** Optical micrograph of etch pits. Each direction was determined by the X-ray analysis. Etching for 5 s in acetone at 18 °C. Scale bar, 30 μm.

unsuccessful. A variety of organic solvents were examined as etchants; acetic acid produced ill-defined patterns, while acetone, ethanol, benzene, carbon tetrachloride, and chloroform produced well defined etch patterns of lozenge shape. In every case, except for acetic acid, pits of about 10 × 10 μm<sup>2</sup> in area were easily produced within a few seconds at 18 °C all over the surface. As shown in Fig. 1, almost all the pits have a common crystallographic direction. The two angles of lozenge-shaped pits were 75° and 105°; the values were the same as those of the crystal habit. The density of the pits was of the order of 10<sup>4</sup> cm<sup>-2</sup>. Two types of pits can be seen from Fig. 1. One is of the inverted pyramidal shape, and the other is of the inverted truncated pyramidal shape.

**Fig. 2** Electron micrographs of two typical pits. Etchant, acetone. The first stage of replica was made by shadowing at 30° with germanium and by supporting normally with carbon. *a*, Two adjacent pyramidal pits; *b*, a flat-bottomed pit. Scale bar, 2 μm.





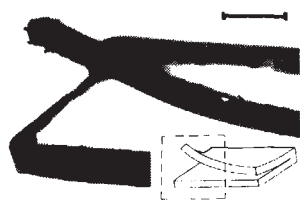


Fig. 3 Optical photograph showing part of the whole cleaved crystal and a sketch of a cleaved crystal. The lower plate, not bent, was used for simultaneous observation. Scale bar, 1 mm.

Transmission electron micrographs around the centre of an etch pit give molecular features of the etching process in detail. As shown in Fig. 2a the pit has several spiral steps whose terminations can be distinguished clearly at the specific points on the pit. These spiral steps also indicate a possible singular centre of the dissolution. Thus the pyramidal pit takes its position at the emergent end of the dislocation perpendicular to the (001) plane. Similar dislocations in stearic acid crystals have already been predicted from the observation of growth spirals on the as-grown (001) surfaces<sup>1</sup>. In Fig. 2b, the flat area, without any etching step, of the pit-bottom can be thought to be molecularly flat. The molecularly flat-bottomed pit can be interpreted by the movement of the dislocation during the etching process; if the dislocation moves out of a pit formed during the first etching, further etching must enlarge the pit in the lateral direction so that the initial pit becomes flat-bottomed. At the same time, the movement of the dislocation would produce a new pyramidal pit at the final position of the dislocation.

If a dislocation, penetrating through the crystal from one surface to another, does not move during the etching process, the pit in direct correlation to such dislocation would grow to be a deeper and larger pyramidal one, while the flat-bottomed pit becomes wider but not deeper, as the etching proceeds. The penetrating dislocation is expected to appear at the cleavage surface. To verify this, the etch pits on the as-grown surface as well as the cleavage surface, (001) plane, of a single crystal were observed simultaneously (Figs 3 and 4). Figure 4 shows the

developments of the two types of pits during their etching process. Two pits of no. 3 and 4, with a rather small-flat bottom at the etching time of 5 s, become large typical flat-bottomed pits in 13 s after the start of etching. On the other hand, each pyramidal pit on the as-grown and cleavage surface becomes deeper and larger, and then each centre of the two pits approaches each other in the projected *ab* plane. This approach can be easily understood because the dislocation line is inclined to the direction of the illumination. Thus it is obvious that two pyramidal pits observed in Fig. 4 lie at the two ends of the dislocation penetrating through the crystal.

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## Can sunspots influence our weather?

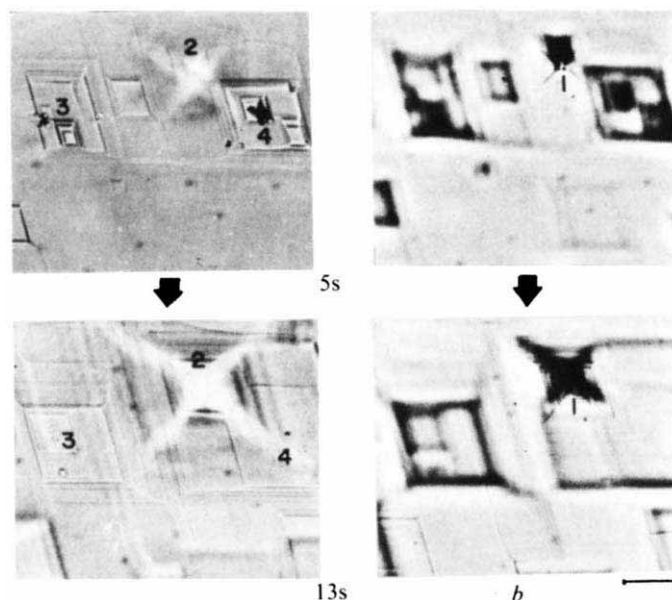
THE discovery of sunspots, in particular the 11-yr cycle, has led to many attempts to detect correlations between the sunspot number and weather changes in the Earth's troposphere<sup>1</sup>. The past decade has seen a renewed interest based on modern data and the concepts of solar-terrestrial physics<sup>2</sup>.

Claims have been made about correlations between H $\alpha$ -flares, crossings of the solar magnetic sector boundaries or sunspots, and tropospheric effects such as low pressure troughs at the 500-mbar level<sup>3</sup>, the influx of stratospheric air masses<sup>4</sup>, thunderstorm activity<sup>5</sup>, geoelectric potential<sup>6</sup> or rainfall<sup>7</sup>. There is no general agreement about the reality of these effects because it is difficult to prove their significance and no acceptable cause and event mechanism has been proposed. Many authors have considered the invisible part of the active Sun as a possible source of these effects because the electromagnetic and corpuscular components of the radiation from the active Sun generate spectacular disturbances in the atmosphere above 60 km. The energies involved in bringing these disturbances about, are only a minute fraction of the solar constant and are, therefore, unlikely to have any direct influence on the troposphere<sup>8</sup>.

King *et al.*<sup>9</sup> have shown that during the last 11-yr solar cycle, the sunspots tended to appear on preferred longitudes on the Sun so that a period of about 27.5 d, the mean period of the solar rotation, is clearly visible in the sunspot number. This is in accord with observations of coronal transients<sup>10</sup>, high-speed solar wind flows<sup>11</sup> and the sector structure of the interplanetary magnetic field<sup>12</sup>. King *et al.*<sup>9</sup> claimed furthermore, that the solar rotation effect in sunspots is correlated with changes in the height of the tropospheric 500-mbar level. The global pressure field displays a wave-like pattern with waves of zonal wave numbers 0, 1 and 2 having maximum amplitudes at higher latitudes<sup>9</sup>.

Although this observation has not been confirmed it suggests a mechanism which may be associated with the visible spectrum of the solar radiation. Radiation from the active Sun, which behaves like a rotating beacon, may influence the transmission of the Earth's atmosphere so that the radiation reaching the ground varies with the same period of  $\sim 27$  d. At least one piece of evidence indicates that the solar constant *S* observed on the ground exhibits such a periodicity with an amplitude of about 0.1% (ref. 13).

Fig. 4 Optical micrographs of the etch pits of pyramidal shape, no. 1 and 2, and of flat-bottomed shape, no. 3 and 4. *a*, taken in focus on the as-grown surface. *b*, taken in focus on the cleaved surface. Etchant, acetone at 18 °C. Scale bar 100  $\mu$ m.





In order to estimate the reaction of the atmosphere to such a small variation of  $S$ , consider an isothermal atmosphere with scale height  $H = 8$  km. The mean energy input per unit mass within the atmosphere due to a change  $\Delta S = 10^{-3} (1 - A) S \approx 1 \text{ W m}^{-2}$  is given by

$$\Delta Q = \frac{\Delta S}{4\rho_0 H} = 2.3 \times 10^{-5} \text{ W kg}^{-1} \quad (1)$$

where  $A = 0.3$  (the albedo),  $\rho_0 = 1.3 \text{ kg m}^{-3}$  (the density at the ground) and  $S = 1.37 \text{ kW m}^{-2}$  (the solar constant).

From the first law of thermodynamics and the hydrostatic law, I estimate a pressure amplitude of

$$|\Delta p| = \frac{\Delta S}{4\omega H} \approx 10 \text{ N m}^{-2} \approx 0.1 \text{ mbar} \quad (2)$$

if  $\omega = 2\pi/27 \text{ d} = 2.7 \times 10^{-6} \text{ s}^{-1}$ . The solar radiation primarily heats the ground, from where it is transported into the atmosphere through turbulent heat exchange. That transport mechanism is mainly effective within the lowest part of the troposphere so that  $\Delta Q$  from equation (1) is probably an underestimation for that part of the atmosphere. The land-ocean coverage is such that global-scale waves including mainly those of zonal wave numbers 0, 1 and 2 are generated and propagate upwards. The prevailing, but fluctuating, westerly wind modifies the horizontal and vertical structure of the planetary waves, and it is clear that the only waves that will be detectable within the meteorological noise by a statistical analysis are those which have vertical wave lengths of the order of, or greater than, the scale height. It can be shown that these waves are of the Rossby-Haurwitz type having maximum amplitudes at higher latitudes.

Detailed calculations (to be published elsewhere) assume a heat source distribution on the ground which reflects the land-ocean distribution<sup>14</sup>. The heat input into the atmosphere by turbulent transport was derived from a diffusion equation<sup>14</sup>. Linear tidal theory was then used<sup>15</sup> to determine the meridional and the height structure of the individual wave modes in the presence of a constant prevailing westerly wind of the order  $10 \text{ m s}^{-1}$ . Only those wave modes were considered which have vertical wave lengths of the order or greater than one scale height. It was shown that for zonal wave number  $m = 2$ , these waves consist of eastward- and westward propagating waves of the Rossby-Haurwitz type. For waves of wavenumber  $m = 1$ , only westward propagating waves of that type exist, so that the waves of zonal wavenumber two probably will dominate. Maximum pressure amplitudes of  $|\Delta p| = 0.3 \text{ mbar}$  near  $\pm 60^\circ$  latitude and on the ground are predicted for waves of wavenumber two. Waves of such small amplitude are at the limit of detectability. But they may be found in analyses such as that carried out by King *et al.*<sup>9</sup>.

The mechanism suggested here may be the primary source of different types of Sun-weather relationship. If short-lived meteorological phenomena such as those associated with solar flares or sector boundary crossings are generated by active longitudes on the Sun, the 11-yr solar-cycle effects may be the result of an 11-yr variation of the amplitude of the 27-d forcing function.

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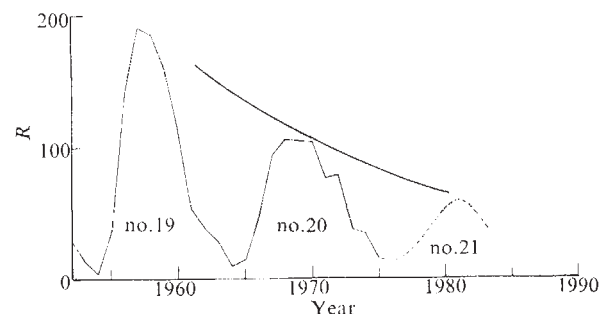
## Equatorial solar rotation and its relation to climatic changes

THE equatorial solar rotation speed, estimated from observations made over the past 10 years of the sunspot longitudinal motions over the solar disk, has shown a tendency to increase as relative sunspot numbers decreased. During these years, covering most of solar cycle no. 20 (1965-76), the magnitude of the solar rotation speed averaged annually showed a good inverse correlation with the annual relative sunspot numbers. I suggest here that this variation of the equatorial solar rotation speed may be responsible for the Earth's present unusual climatic conditions.

The highest speed was observed for 1976, when the observed sunspot activity was at its lowest since 1967, except for 1974, and was approximately equal to that estimated for the year 1643, just before the beginning of the Maunder Minimum (1645-1715), during which very few sunspots were reported. According to Eddy *et al.*<sup>1</sup>, there is evidence that, during the Maunder Minimum, the speed of the equatorial solar rotation, averaged annually, was larger by 4% or more than that now observed<sup>2</sup>. They have based this assumption on analysis of the sunspot observations made by Scheiner, Hevelius and others in the seventeenth century (see ref. 3 for details). Although the causal relationship between the degree of the development of solar activity and the pattern of this solar rotation speed was not known, their result suggested that, during the period when this solar activity was higher relative to the averaged one, this degree of development, as indicated by the relative sunspot numbers, for instance, must be lower than that observed for any other period when this speed is relatively low<sup>2</sup>. Furthermore, from their results, it could be predicted that the severe climatic conditions experienced during the Maunder Minimum might have been related to the strong depression of solar activity and hence with the increase of the solar rotation speed.

Based on observations of the motion of sunspot groups over the solar disk, Howard<sup>4</sup> has measured the yearly variation of the mean speed of the solar rotation as a

**Fig. 1** The relative sunspot numbers  $R$  since 1952. These numbers, often called the Wolf numbers  $R$ , are published annually by the Zurich observatory, based on the reports on sunspot numbers from observatories throughout the world. These numbers are counted by using the formula,  $R = k(10g + f)$ , where  $k$ ,  $g$  and  $f$  are, respectively, the seeing factor, the numbers of sunspot groups and of individual sunspots. A curve predicting these numbers for the cycle no. 21 is indicated by a dotted line (see text). A heavy line shows the general trend of the sunspot activity for the three cycles nos 19-21.



function of the solar latitude for 1967–76. He found that this speed has gradually increased throughout this period until 1976, when the sunspot activity was at minimum. Furthermore, it should be noted that the speeds observed during the above period were all relatively greater than those estimated by Ward<sup>2</sup> for the earlier period.

Although it is not clear whether this tendency will continue for the coming cycle no. 21, it seems likely that the whole pattern will still show the same tendency as observed in the last cycle no. 20 (1965–76), even though it might slow down slightly during the rising portion of this cycle (1977–79). As suggested in Fig. 1, the relative sunspot number for the next maximum is expected to be 20 or so less than that observed in the last cycle, because the solar activity as a whole has tended to decrease since cycle no. 19 as predicted from both the observed progression of the 80-year quasi-periodic variation of the relative sunspot numbers and the relatively slower start of the present cycle no. 21.

In order to discover whether there is a causal relationship between the sunspot activity and the solar rotation speed, the values of this speed at the equator observed by Howard<sup>4</sup> have been plotted as a function of the relative sunspot numbers for the years from 1967 to 1976. Figure 2 clearly shows that the magnitude of this equatorial rotation speed has an inverse relationship with these sunspot numbers. Thus, the solar activity as a whole tends to decrease with the increase of the speed of the equatorial solar rotation during the above period.

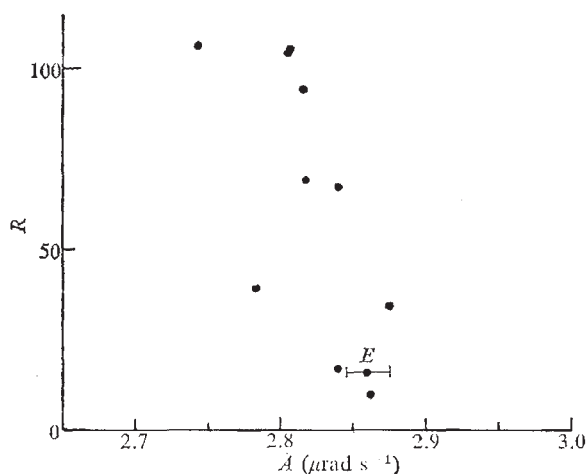


Fig. 2 The equatorial angular velocity  $A$  of the solar rotation as a function of the relative sunspot numbers  $R$  for the years from 1967 to 1976 (●, average velocity). (The data are from ref. 4.)  $E$  with error bar shows the velocity for 1643, two years before the beginning of the Maunder Minimum<sup>1</sup>.

If the sunspot numbers during cycle no. 21 vary as predicted in Fig. 1, the tendency shown in Fig. 2 would continue for this cycle because of the lower sunspot activity; that is, it is possible that this speed will increase to the values observed during the Maunder Minimum.

In Fig. 2, the equatorial rotation speed for 1643, estimated by Eddy *et al.*<sup>1</sup>, is also plotted as a comparison with the present trend. By examining the observed speed for 1643, the range of this speed has been estimated and shown with an error bar in Fig. 2. The mean value of this speed is almost the same as that observed in 1976, though the relative sunspot numbers are slightly different. Hence, it seems possible that the general trend of the variation of the equatorial rotation speed will be maintained during the new cycle if the relative sunspot numbers for these years behave as predicted in Fig. 1.

Climatic conditions were very severe during the Maunder Minimum as recorded in London, for instance<sup>3,5</sup>. When the observations from this period are compared with the corresponding climatic conditions, it becomes possible to conclude that the rare severe depression of the solar activity (and the consequent speeding up of the equatorial solar rotation) was responsible for this little ice age for 70 years or more<sup>3</sup>.

Figures 1 and 2 and the discussion on the Maunder Minimum, indicate that the Earth's present unusual climate might be associated with the decreasing trend of the sunspot activity (Fig. 1) and the related increase in the equatorial solar rotation speed—the speed at the equator of the solar differential rotation. It therefore seems likely that the present unusual climatic conditions will remain as long as the solar activity continues to decrease.

When we consider both the photospheric magnetic field and the high-speed flow of the solar wind during the past few years, it is clear that both the large-scale coronal hole and the apparent width of this high-speed flow region have been steadily growing<sup>6,7</sup>. Their steady growth strongly indicates that the decrease of the solar activity is likely to continue for the coming cycles, continued observations would give us important clues as to the Earth's future, in particular, to its climate. It may also be possible to study possible causal relationship between the Earth's climate and the solar activity variations in the long term.

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## Reproductive effort and life history strategy of the Aldabran giant tortoise

THE Aldabra giant tortoise, *Geochelone gigantea* Schweigger, has a large body size (19–120 kg)<sup>1</sup>, long life span (65–90 yr)<sup>2</sup>, late maturity (females 17–23 yr)<sup>3,4</sup> and iteroparity<sup>3</sup>. The three isolated island populations of different densities on Aldabra show dissimilarity in their reproductive parameters (for example mean clutch size, mean egg weight, proportion of mature females breeding and the number of clutches per female per year) and in the variation of these parameters from year to year through annual changes in rainfall<sup>3</sup>. Moreover, their reproductive response to these changes in rainfall is rapid; they have a relatively high maximum (potential) rate of natural increase (maximum clutch size 28)<sup>3</sup> and mature females in the high density population are half the weight of those in the low density area<sup>2,4</sup>. I have postulated that the reproductive effort would differ between the different density populations and that this effort would change in relation to changes in food availability. This contention is supported by the results reported here.

I examine here the apportionment of the assimilated resources towards reproduction, growth and respiration and the selective pressures which give rise to this allocation of energy. Assimilation ( $A$ ) is defined as the sum of production due to reproduction ( $P_r$ ), production due to growth ( $P_g$ ) and respiration ( $R$ ). After the onset of sexual maturity, growth is very slow (ref. 2 and my unpublished results) and for the purposes of this work is taken to be zero. Thus  $P_r/A \approx P_r/(R + P_r)$  and this ratio will be used to measure reproductive effort.



Table 1 Reproductive effort compared with population density and years

|                                         | Very low density (5 per ha)*<br>1976 | Low density (7 per ha)*<br>1975 | High density (27 per ha)*<br>1975 | 1976  |
|-----------------------------------------|--------------------------------------|---------------------------------|-----------------------------------|-------|
| Mean weight of mature female (g)†       | 48,300                               | 46,700                          | 19,300                            |       |
| Reproductive output (g per ♀ per yr)‡   | 4,204                                | 2,303                           | 91                                | 303   |
| Reproductive output (kJ per ♀ per yr) § | 29,786                               | 16,317                          | 645                               | 2,147 |
| Annual respiration (kJ per ♀ per yr) ¶  | 470,323                              | 455,905                         | 201,864                           |       |
| Reproductive effort (%)                 | 6.0                                  | 3.4                             | 0.3                               | 1.0   |

\*Figures taken from ref. 1.

†Maturity was determined by dissection<sup>3</sup>.‡Mean clutch size × mean egg weight × mean clutches per ♀ per yr<sup>3</sup>.

§Calculated using energy value per g dry weight of eggs excluding the shell (my work with N. K. Jenkins, in preparation).

¶Assuming 7 h activity (feeding) and 17 h inactivity (sleeping) per 24 h (from observation) (my unpublished results). Oxygen consumption in ml per animal per h (ref. 11) is 140.8 (individual weight<sup>0.97</sup>) for active animals and 45.47 (individual weight<sup>0.82</sup>) for inactive animals. Calorific equivalent for 1 ml of oxygen is 5.05 where respiratory quotient is unity<sup>11</sup>.

||Mean reproductive output/(mean reproductive output + mean annual respiration).

This index is subject to several objections as it does not take into account the energy expended in growth in the annual energy budget (albeit low), finding or digging a suitable place for egg deposition, possible differences in metabolic rates between individuals from the three populations or possible variations in the energy costs of egg production. The care invested in a single offspring was estimated by the average egg weight as there is no parental care after oviposition. Recognising these difficulties, I feel that the methodology is adequate to allow discussion of the differences in reproductive effort and the life history adaptations of this large terrestrial herbivore.

Table 1 shows the reproductive effort compared with population density during the 2 yr of study. Reproductive effort increased as the population density decreased. The rainfall increased by approximately 40% in 1976 compared with 1975 and there was a greater proportional increase in reproductive effort in the high density population than in the low density population through an increase in food resource availability.

One would not expect any increase in reproductive effort by the low density population if it was food resource limited. But both the very low density and low density populations are expanding<sup>1,3</sup>. The apparently greater sensitivity of the high density population to changes in food resource availability (as represented by rainfall) suggests they are closer to the carrying capacity (that is available food) than in the low density population. The low density population is mainly limited, probably much below the level set by food availability, by high levels of nest destruction by nesting females caused by the severe lack of suitable nesting areas<sup>3</sup>. Indeed the increase in reproductive effort by the low density area females occurred through increasing mean egg weight, whereas in the high density area mean clutch size, mean egg weight and the proportion of mature females breeding all increased (ref. 3 and my work with M. Coe, in preparation). This was the only available 'tactic' left to the low density area females in order to increase their effort, as any increase in nest numbers would involve the destruction of an equivalent number of existing nests and an increase in clutch size would have a similar effect. In both years of study the number of nests remained the same<sup>3</sup>.

In the high density population mortality is caused mainly by thermal stress<sup>1</sup>. Individual tortoises must spend the middle of each day (approximately 1000–1600 h) shading<sup>4</sup> and, as the distance between shade points particularly on the coast (with a high density population during the rains (ref. 4 and my unpublished results)) prohibits movement by exposing the animal to probable death, their feeding activities are confined to a specific area. Only during short (2–3 d) and infrequent periods of dull cloudy weather can the tortoise move more freely. When the Sun abruptly returns after these periods large numbers of recently dead animals are found well away from any available shade (my unpublished results). It can be predicted that as the rainy season gives way to the dry season (with far fewer dull weather periods) food availability will decrease. Eventually the local population within these shade-focussed feeding areas must exceed the food availability and it is at this time of year that tortoises will move away from these areas in large numbers, presumably risking death through thermal stress rather than slow but inevitable malnutrition. This, mainly circum-

stantial, argument together with other observations on reduced body size<sup>1,2</sup>, retarded growth<sup>2,3</sup>, very low recruitment<sup>3</sup>, pre-ovulatory follicular atresia<sup>3</sup> and the ratio of estimated to predicted production<sup>5</sup> supports the suggestion that the population in the high density area is at the carrying capacity determined by food.

The low density population is in a relatively less food-limited environment and shows early maturity and large clutches of large eggs; whereas the high density population, living in a more food-limited environment, shows delayed maturity and small clutches of small eggs. Most of these parameters could be predicted if the position of the populations with respect to resources were the primary determinants of the evolution of life history characters. The advent of large eggs in the low density population, however, was not predicted as the eggs in the very low density and high density populations were both small<sup>3</sup>. Large eggs produce large hatchlings which survive better than small hatchlings<sup>3</sup>. Giant tortoise hatchlings for the first 2 or 3 yr of life feed in areas where, because of the terrain, animals older than 4–5 yr cannot feed (my unpublished results). It was argued<sup>3</sup> that these very young individuals had greater difficulty in finding suitable food in the low density area than in the other two areas and therefore the larger eggs were incorporated into the life history adaptation. Evidence for this hypothesis was inferred from observing that hatchlings in the high density area grew more than those in the low density area during the first year<sup>3</sup>.

It becomes apparent when considering the high maximum rate of natural increase, the quick response to changes in food availability, the larger hatchlings in the less-limited low density area and the lower mean weight of mature adults in the high density area that the Aldabran giant tortoise populations highlight the inadequate and over-simple concept of *r* and *K* selection<sup>6</sup>. The low mean weight of mature females in the high density area together with the ability to make a rapid and potentially large increase in reproduction may have been selected in order to effect a compromise between parental risk (in making resources available to the offspring) and food availability. By having a smaller body size, a greater proportion of any increase in food availability can be used in reproduction without increasing, unacceptably, the risk to a parent's lifetime reproductive success.

A recent paper<sup>7</sup> on the problem of 'temporally dynamic reproductive strategies' viewed in terms of the conceptual framework of *r* and *K* selection concluded that mean *r*-*K* continuum<sup>8</sup> positions have relatively little explanatory value where a measure of year-to-year variation in reproductive effort is more relevant in considering life history strategies and selective pressures. Moreover it was suggested that the proportion of mature females breeding can be used to infer variation in population reproductive effort. In the case of the giant tortoise the increase in reproductive effort in the low density population would be overlooked using this index because it occurred through an increase in mean egg weight and not an increase in breeding females. Additionally in the high density population not only did the proportion of mature females breeding increase but mean clutch size and mean egg weight also increased. Other work<sup>9,10</sup> has emphasised the need to consider the environmental dimensions essential to provide complete understanding of the evolution of life histories. It is hoped that this



example within a species clearly demonstrates some of these dimensions.

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## Behaviour of adult and immature male baboons during inter-group encounters

SOCIAL interaction between groups of Old World monkeys varies from peaceful mingling to marked aggression<sup>1-3</sup>. One constant feature, however, concerns the differing behaviour of immature and adult males; and these differences have been related to differences in male reproductive success at different stages of the life cycle. Immature and young adult males in multi-male groups of baboons and macaques frequently approach and interact with individuals outside their natal group, sometimes transferring from one group to another<sup>4-7</sup>. Two suggested causes of such group transfer are inability to gain access to oestrous females in the natal group and attraction to oestrous females in other groups<sup>8</sup>. In contrast, adult males—both in species with one-male social units and in species with multi-male groups—are frequently aggressive during intergroup encounters, often chasing or 'herding' members of their own group away from other groups<sup>7,9,10</sup>. Herding usually involves high-ranking adult males who are also the individuals who copulate most often<sup>10,11</sup>. Males are most likely to herd adult females, but rarely herd females who are lactating<sup>10-12</sup>. (Although occasional herding in gelada baboon one-male units has been reported<sup>13</sup>). Herding has been suggested both to mobilise individuals before group movement<sup>7</sup> and to maintain a male's access to oestrous females over time<sup>10</sup>. To investigate the function of herding we observed the intergroup encounters of one multi-male group of baboons for 15 months. Our observations suggest not only that herding is correlated with differences in male reproductive success but also that it is related to changes in the female reproductive cycle and to long-term bonds between particular males and females.

We studied the intergroup encounters of a group of southern African baboons (*Papio cynocephalus ursinus*) consisting of two adult males, eight adult females, and between 14 and 20 immatures. During 15 months' observation, this group came to within 500 m of another group on 174 occasions, with such 'encounters' lasting from 15 min to 16 h (the latter occurred when two groups occupied adjacent sleeping sites). Although the study group regularly came into contact with three different groups, and behaved similarly during encounters with each, 77% of all encounters involved one group of approximately 50 individuals.

During 44% (77) of all encounters, one or more of the group's five subadult males (estimated age 44-58 months)

sat at the boundary between groups. In addition, during the 4 months preceding their emigration, at least one of the subadults exchanged affiliative gestures with the other group's subadult males and/or copulated with the other group's females on 59% (13) of all encounters. Copulation occurred when oestrous females from the neighbouring group approached the study group and presented to both adult and immature males. Although the females were occasionally herded away by the adult males of their group, such males never threatened males in the study group<sup>10,14</sup>. More often, males in the study group were able to respond to the females' presents. Immature males and the subordinate adult male were more likely than the alpha male to receive presents, and subadult males were the most likely to respond to them (Table 1). When four of the subadult males emigrated from the study group, they did so following an encounter which included copulations, and they left in the company of oestrous females from the neighbouring group.

Concurrently, more than 90% (159) of all encounters were accompanied by at least one 'bout' of herding by an adult male: separate bouts were defined as any instance of herding separated by an interval of at least 1 min when herding did not occur. The alpha male, who accounted for the largest single proportion of copulations<sup>15</sup> and fertilised six of the eight females who became pregnant<sup>16</sup>, was responsible for 85% of all herding bouts. Immature males, in contrast, accounted for less than 1% of all observed herding bouts.

**Table 1** Proportion of presents by oestrous females from neighbouring groups to which males responded, either by touching, grooming, or copulating with the presenting female

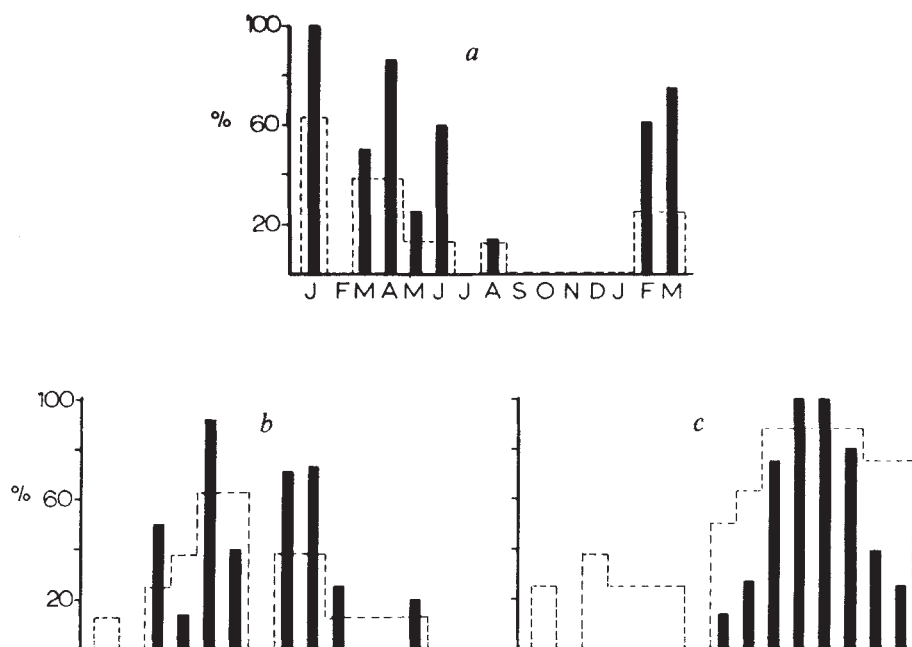
|             |             | No. of presents received | % Presents responded to |
|-------------|-------------|--------------------------|-------------------------|
| Adult ♂♂    | Alpha       | 0                        | —                       |
|             | subordinate | 20                       | 10                      |
| Subadult ♂♂ | A           | 10                       | 40                      |
|             | C           | 8                        | 50                      |
|             | K           | 4                        | 50                      |
|             | S           | 3                        | 67                      |
|             | L           | 17                       | 59                      |

During herding, the male involved chased one or more female around and through the group, repeatedly giving loud double-barks<sup>10,17</sup>. Of 200 herding bouts, 81% (161) began with the chasing of one adult female. During the course of herding, however, other animals were often supplanted by the male's approach and they, too, could be herded. Herding seemed to cause great anxiety among females and immatures, who screamed, raised their tails, and fear-grinned as they were herded. The net effect of herding was to drive most adult females, and many immatures near them, away from the other group. Herding was therefore both an aggressive, disruptive behaviour and a behaviour which tended to promote intragroup cohesion by maintaining spatial separation between groups.

Throughout the study, there was a variable number of females each month either sexually cycling, pregnant, or lactating. Taking data from the 150 occasions when the alpha male 'chose' only one female for herding, Fig. 1 shows that this male herded sexually cycling females more often, and lactating females less often, than would have been expected had females been 'chosen' at random.

The amount of herding received by each female thus depended on both the ranging behaviour of her own and other groups, and her reproductive state when intergroup encounters occurred. It was also possible to test for individual preferences on the part of each adult male and to

**Fig. 1** Proportion of all adult females who were either (a) sexually cycling, (b) pregnant or (c) lactating each month (broken lines), compared with the proportion of the alpha male's 'choices' for herding each month that involved females in each of these reproductive states (solid histograms). Sexually cycling females were chosen more often than expected in eight out of eight possible months, pregnant females more often in six out of 11 months, and lactating females more often in 2 out of 13 months



compare these preferences with the males' long-term bonds with particular adult females.

Examination of social interactions between the two adult males and eight adult females throughout the reproductive cycle indicated that, whereas each male formed sexual consortships with a number of females at the height of oestrus<sup>16</sup>, males and females also showed individual preferences which persisted during pregnancy and lactation<sup>18</sup>. Table 2 presents data on the relative frequency with which each adult male herded each female in different reproductive states. Results indicate that, in all reproductive states, males were most likely to herd females with whom they had the closest long-term social bonds. Each male's preference for one specific individual often overrode preference for females in particular reproductive states, as, for example, when the alpha male repeatedly herded his preferred partner even though she was lactating and there were other, sexually cycling females in the group.

Disparities in the behaviour of adult and immature males may be related to the males' differing access to oestrous females at different times during their life cycle. Adult males in the study group accounted for the great majority of copulations—particularly around the time of ovulation—

and seemed to limit the access of immature males to sexually receptive females<sup>15</sup>. As a result, immature males may have been more attracted than adult males to sexually receptive females in neighbouring groups.

The alpha male's 'choice' of particular females for herding had two important consequences. First, in both the short and the long term, it served potentially to increase his reproductive success by isolating from males in other groups (1) those females who were about to ovulate, and (2) those females in whom the male would invest the greatest direct social support in future. Male herding of sexually receptive females was therefore both behaviourally and functionally similar to the herding of oestrous females in intragroup interactions observed in a previous study of a multi-male group of baboons<sup>14</sup>. Second, male choice of particular females seemed to minimise the potentially disruptive effect of herding. As noted earlier, each male was most likely to herd the female with whom he had the closest long-term social bond. Consequently, herding most often involved those individuals who were most likely to follow agonistic behaviour with relaxed proximity or grooming<sup>18</sup>. Males therefore seemed to be able to maintain separation between groups with a minimum of social disruption.

**Table 2** Ranked frequencies with which each female was 'chosen' for herding by each adult male in each reproductive state

| ♀♀ | Alpha male (n = 150) |          |           |              | ♀♀ | Subordinate male (n = 11) |          |           |              |
|----|----------------------|----------|-----------|--------------|----|---------------------------|----------|-----------|--------------|
|    | Sexually cycling     | Pregnant | Lactating | Sum of ranks |    | Sexually cycling          | Pregnant | Lactating | Sum of ranks |
| H  | 2                    | 7        | 6.5       | 15.5         | H  | 5                         | 5.5      | 5.5       | 16.0         |
| W  | 6.5                  | 6        | 4         | 16.5         | W* | 1                         | 1        | 1.5       | 3.5          |
| L* | 1                    | 1        | 2         | 4.0          | L  | 5                         | 5.5      | 5.5       | 16.0         |
| S  | 6.5                  | 5        | 6.5       | 18.0         | S  | 5                         | 5.5      | 5.5       | 16.0         |
| LP | 6.5                  | 4        | 3         | 13.5         | LP | 5                         | 5.5      | 5.5       | 16.0         |
| PM | 4                    | 8        | 1         | 13.0         | PM | 5                         | 5.5      | 1.5       | 12.0         |
| M  | 3                    | 2        | 5         | 10.0         | M  | 5                         | 2        | 5.5       | 12.5         |
| P  | 6.5                  | 3        | 8         | 17.5         | P  | 5                         | 5.5      | 5.5       | 16.0         |

n, Number of herdings involving each male and one female.

\*Each male's 'preferred' female<sup>16,18</sup>.

Relative frequencies with which each female was chosen by each male in each reproductive state calculated as follows: for example, for the alpha male and female H during H's sexual cycle

$$\frac{\text{total times alpha male 'chose' } \text{♀H during } \text{♀H's sex. cyc.}}{\text{total times alpha male 'chose' any female during } \text{♀H's sex. cyc.}}$$

For every female, at least five bouts of herding occurred during each reproductive state.

Finally, the pattern of male herding reported here for a multi-male group of baboons bears several similarities to herding by hamadryas baboon males in one-male units<sup>10</sup>. Results thus support the suggestion<sup>18,19</sup> that there may be marked similarities in the pattern of social relationships within one-male and multi-male social groups despite the more obvious differences in group size and composition.

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## Nitrate reductase-deficient mutants in barley

MUTANTS have long been used to study genetic and biochemical regulation of metabolic pathways in microorganisms. Often it is a relatively simple task to screen large bacterial populations for an individual lacking a specific enzyme or enzyme product. Defined enzyme mutants would also be valuable research tools for plant geneticists and biochemists. Unfortunately, very few biochemically defined mutants have been selected in higher plants, principally because of the genetic and biochemical complexity, and the long generation time of higher plants<sup>1</sup>. But, these obstacles could be overcome in plants by inducing high mutation frequencies and developing effective techniques for selecting biochemically defined mutants. High mutation frequencies can be readily induced in some higher plant species by sodium azide, a potent base substitution mutagen in barley<sup>2,3</sup>. Nitrate reductase, which has an important function in higher plants<sup>4</sup>, has been extensively studied in bacteria and fungi<sup>5</sup>, is highly regulated<sup>6,7</sup>, and has several catalytic activities including NAD(P)H, FMN<sub>2</sub> and reduced methyl viologen nitrate reductase activities, and NAD(P)H cytochrome *c* reductase activity<sup>8</sup>. Nitrate reductase is therefore an excellent enzyme in which defined mutants in higher plants can be selected, studied, and compared with microorganisms. We describe here a procedure for the induction and selection of nitrate reductase-deficient mutants in higher plants, and the partial characterisation of three nitrate reductase-deficient mutants in barley.

Nitrate reductase-deficient mutants were induced with sodium azide in 'Steptoe' barley (*Hordeum vulgare* L.) as described by Kleinhofs *et al.*<sup>7,8</sup>. Steptoe barley seeds were hydrated at 0–2 °C overnight and then germinated at 20 °C in vigorously aerated water for 8 h. The seeds were then placed in an aerated solution containing 1 mM sodium azide and 0.1 M phosphate buffer (pH 3.0) for 2 h. The treated seeds were washed for 30 min, blotted and dried in a fume hood. The dry seeds were planted in an

isolated field plot. The crop produced (M<sub>2</sub> seed) was harvested in bulk.

The nitrate reductase-deficient mutants were selected from the M<sub>2</sub> segregating population by qualitatively estimating *in vivo* nitrate reductase activity. Individual 7-d-old control or segregating M<sub>2</sub> seedlings with normal green appearance were screened for the presence of nitrate reductase activity by using a modification of the *in vivo* assay described by Finke *et al.*<sup>9</sup>. Approximately 8 cm of the primary leaf was excised, cut in half, and placed in a test tube containing 4 ml of an incubation medium containing 0.1 M potassium nitrate, 0.1 M potassium phosphate (pH 7.0) and 0.01% Tergitol NPX. The leaf tissue was then vacuum infiltrated and incubated in the dark at 30 °C; nitrite was then determined as previously described<sup>9</sup>. Nitrate reductase-deficient seedlings were transplanted to soil and fertilised with ammonium sulphate.

Nine nitrate reductase-deficient seedlings were selected from approximately 6,000 M<sub>2</sub> seedlings tested. None of the 5,000 control seedlings tested was nitrate reductase-deficient. Five of the nine nitrate reductase-deficient selections were sterile or otherwise failed to produce seed. Progeny tests of the four selections confirmed that three (Az12, Az13, Az23) are nitrate reductase-deficient (Table 1).

These mutants have low *in vivo* nitrate reductase activity and low *in vitro* nitrate reductase activity when either NADH or FMN<sub>2</sub> is used as an electron donor. All three mutants are also similar in that they have elevated leaf nitrate levels and nitrite reductase activities. But they differ in that Az12 and Az23 have low cytochrome *c* reductase activities whereas Az13 has cytochrome *c* reductase activity similar to the control. Similar results were obtained with field grown plants except that the nitrate content of the leaves of the nitrate reductase mutant plants was much greater than in the control (Table 2). In spite of the low nitrate reductase activity, the mutant selections seem to be as vigorous as, and morphologically indistinguishable from, the control.

The higher levels of nitrate in the mutants is presumably the result of the lowered capacity of the mutants to reduce nitrate. The high levels of nitrite reductase in the mutants (Table 1) may be caused by the high levels of nitrate inducer, although the levels of nitrate in the control are well above those generally considered necessary to give optimum induction of nitrate reductase<sup>10</sup>. Alternatively, the mutants may have lower levels of reduced nitrogen available for the repression of nitrite reductase synthesis. Ammonium and other forms of reduced nitrogen have been reported to repress nitrate reductase synthesis<sup>6</sup> and would be expected to have a similar effect on nitrate reductase.

The different levels of cytochrome *c* reductase activity in the mutants seems to be the result of different muta-

**Table 1** Nitrate, nitrite, cytochrome *c* reductase activities, and nitrate content of 7-d-old nitrate reductase mutant and control seedlings

| Selection | NRA  |                  |                | NiRA | CRA | NO <sub>3</sub> <sup>-</sup> |
|-----------|------|------------------|----------------|------|-----|------------------------------|
|           | NADH | FMN <sub>2</sub> | <i>in vivo</i> |      |     |                              |
| Az12      | 0.4  | 0.0              | 0.2            | 315  | 43  | 1,806                        |
| Az13      | 0.4  | 0.0              | 0.3            | 355  | 242 | 1,657                        |
| Az23      | 0.3  | 0.0              | 0.3            | 370  | 36  | 2,492                        |
| Control   | 27.2 | 28.8             | 4.8            | 197  | 194 | 1,246                        |

Nitrate reductase (NRA) was assayed *in vitro* with NADH as electron donor (ref. 15), with FMN<sub>2</sub> (ref. 16) and assayed *in vivo* (ref. 9). Nitrite reductase (NiRA) and nitrate content were determined as in ref. 15 and cytochrome *c* reductase (CRA) as in ref. 12. Enzyme activities are expressed as  $\mu\text{mol per g of fresh weight per h}$  and nitrate content as  $\mu\text{g NO}_3\text{--N per g fresh weight}$ . Plants were grown in a growth chamber at 20 °C and continuous light for 7 d as described by Warner and Kleinhofs<sup>15</sup>.



**Table 2** Nitrate, nitrite and cytochrome *c* reductase activities, and nitrate content of field grown (heading stage) nitrate reductase mutants and control plants

| Selection | NRA  |                | NiRA | CRA | NO <sub>3</sub> <sup>-</sup> |
|-----------|------|----------------|------|-----|------------------------------|
|           | NADH | <i>in vivo</i> |      |     |                              |
| Az12      | 0.3  | 0.8            | 604  | 114 | 1,007                        |
| Az13      | 0.4  | 1.6            | 625  | 288 | 1,135                        |
| Control   | 19.6 | 10.0           | 226  | 174 | 41                           |

tional events. It has previously been reported that nitrate reductase also has cytochrome *c* reductase activity<sup>11,12</sup>. These results suggest that Az12 and Az23 mutations inactivate or prevent the synthesis of the nitrate reductase enzyme including the cytochrome *c* reductase component whereas Az13 seems to have the cytochrome *c* reductase portion of the enzyme still functional. Wray and Filner<sup>12</sup> reported that the cytochrome *c* reductase associated with barley nitrate reductase is induced by nitrate whereas two other cytochrome *c* reductases are constitutive and not associated with nitrate reductase. When grown on an ammonium nitrogen source, Az12, Az13 and the control have very low levels of nitrate reductase and approximately the same cytochrome *c* reductase activities (Table 3).

**Table 3** Induction of nitrate and cytochrome *c* reductase activities in 12-d-old nitrate reductase mutant and control seedlings

| Selection | N source                     | NRA  | CRA | NO <sub>3</sub> <sup>-</sup> |
|-----------|------------------------------|------|-----|------------------------------|
| Az12      | NO <sub>3</sub> <sup>-</sup> | 0.3  | 29  | 2,506                        |
|           | NH <sub>4</sub> <sup>+</sup> | 0.0  | 40  | 13                           |
| Az13      | NO <sub>3</sub> <sup>-</sup> | 0.3  | 343 | 2,579                        |
|           | NH <sub>4</sub> <sup>+</sup> | 0.1  | 46  | 19                           |
| Control   | NO <sub>3</sub> <sup>-</sup> | 30.5 | 300 | 1,545                        |
|           | NH <sub>4</sub> <sup>+</sup> | 1.2  | 49  | 9                            |

When induced with nitrate, Az13 and control seedlings have a nitrate-inducible cytochrome *c* reductase which is presumably associated with the nitrate reductase protein. Based on work with nitrate reductase mutants in *Aspergillus nidulans*<sup>19</sup> and *Neurospora crassa*<sup>11</sup>, it may be possible to predict the nature of the barley nitrate reductase mutants. Mutations in the regulator loci in fungi<sup>11,13</sup>, result in the loss of all of the nitrate reductase catalytic functions and of nitrite reductase as well. Since all of the barley nitrate reductase mutants contain nitrite reductase then these mutations seem not to be in the regulator locus unless nitrate and nitrite reductases are controlled by separate regulator loci in higher plants. Fungi nitrate reductase structural mutants generally have nitrite reductase activity and in some cases nitrate-inducible cytochrome *c* reductase activity<sup>11,13</sup>. Therefore, Az12 and Az13 are probably nitrate reductase structural mutants since both mutants lack the nitrate reductase catalytic functions and have nitrite reductase activity.

In addition to mutations in the nitrate reductase structural and regulator loci, mutations in the loci controlling the molybdenum component of nitrate reductase also result in the loss of nitrate reductase activity<sup>13</sup>. In *Aspergillus* the molybdenum component mutants have nitrite reductase activity and either a constitutive or an inducible cytochrome *c* reductase activity<sup>14</sup>. The mutants having the inducible cytochrome *c* reductase usually have low levels of nitrate reductase similar to Az13 (Table 3). Therefore, Az13 may be either a nitrate reductase structural mutant or a molybdenum component mutant assuming the barley and *Aspergillus* systems are analogous. Whether these nitrate reductase mutants are in the structural protein for nitrate reductase or some other

locus is not known. More mutants and subsequent analyses are needed to adequately resolve this question.

The induction and selection of defined enzyme mutants such as the nitrate reductase mutants described, should be of considerable help in resolving genetic and biochemical control mechanisms of metabolic pathways in higher plants. Nitrate reductase has received considerable attention in the past 20 years but the work in higher plants has been severely handicapped by the lack of sufficient genetic diversity. Nitrate reductase-deficient mutants have previously been identified in *Arabidopsis thaliana*<sup>17</sup> and now in barley. These mutants are an important first step for studying the genetic regulation of nitrate reductase in higher plants.

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## Possible *in vitro* test for screening drugs for activity against *Babesia* and other blood protozoa

THE normal method of screening compounds for activity against *Babesia* infections of animals is based in the first instance on testing the compound in laboratory rats or mice infected with *B. rodhaini* using a regimen similar to that described for the testing of anti-malarial compounds<sup>1</sup>. Most compounds screened against *B. rodhaini* in this way show no activity at all, therefore there would be justification for developing an *in vitro* test which would greatly reduce the time, cost and numbers of animals involved. We describe here such a test which is applicable to all species of *Babesia* and which exploits the finding that *Babesia* parasites readily incorporate hypoxanthine in an *in vitro* culture system<sup>2</sup>.

The parasites used were *B. rodhaini*<sup>3</sup> maintained in intact LACG mice, *B. divergens*<sup>4</sup> and *B. major*<sup>5</sup> maintained in splenectomised calves, and a *Babesia* sp. (probably *B. ovis*) recently isolated from Scotland<sup>6</sup> and maintained in splenectomised sheep.

Blood from infected and similar non-infected animals was set up in a series of flasks containing culture medium and a test compound. Appropriate controls were included. Cultures were incubated for 1 h and <sup>3</sup>H-hypoxanthine was then added to all flasks, which were incubated overnight. <sup>3</sup>H-hypoxanthine incorporation was measured the next day by liquid scintillation counting<sup>7</sup>. The amount incorporated

by *Babesia* parasites was obtained by subtracting the count of non-infected blood from that of infected blood.

Various compounds were tested and those that were active against *Babesia* parasites inhibited the uptake of  $^3\text{H}$ -hypoxanthine, whereas inactive compounds did not (Table 1). Any compound which reduced the uptake of  $^3\text{H}$ -hypoxanthine by about 75% or more was considered to be active.

In addition to detecting active compounds, the test also indicated which drugs were most effective against particular species of parasite. Of the babesicides tested, Imidocarb and Amicarbalide were the most active *in vitro* against *B. rodhaini* and *B. divergens*, and Diminazene was the most active against *B. major* and "*B. ovis*". These findings agree with *in vivo* studies (refs 8–10 and D. W. Brocklesby, personal communication). Diminazene, however, was ineffective *in vitro* against *B. rodhaini* and *B. divergens* at the concentration used, although in a subsequent experiment a tenfold increase in drug resulted in >95% reduction in  $^3\text{H}$ -hypoxanthine uptake. This drug was also less effective *in vivo* against these parasites<sup>8,9</sup>. *In vivo* studies with Trypan blue have shown that it is principally active against 'large' babesias<sup>11</sup> and ineffective against *B. ovis*<sup>10</sup>, *B. major*<sup>12</sup> and the small parasites; similar findings were recorded in the present work. The antimalarial drug, chloroquine sulphate, was ineffective *in vitro* against the three parasites screened; similar lack of activity was recorded *in vivo*<sup>13</sup>.

Hypoxanthine itself blocked the uptake of  $^3\text{H}$ -hypoxanthine, and both 6- and 2-mercaptapurine (structural analogues of hypoxanthine) seemed to be active. *In vivo* tests with these two compounds showed that they delayed time to death in *B. rodhaini*-infected mice by about 14%

(our unpublished observations). Their activity is probably explained on the basis of competition with hypoxanthine for the enzyme hypoxanthine guanine phosphoribosyl transferase<sup>14</sup>. Activity of 6-thioguanine can be similarly explained. Occasionally more  $^3\text{H}$ -hypoxanthine was incorporated by treated samples than by controls. This could be as a result of drug-induced changes, such as increased parasite or erythrocyte permeability, or because of increased parasite metabolism, but such factors have not so far been investigated.

A small amount of  $^3\text{H}$ -hypoxanthine is incorporated by non-infected blood, principally by white cells. This amount is insignificant when compared with the massive uptake by *Babesia* parasites<sup>2</sup>. By subtracting counts of control blood from those of infected blood such 'background' was largely eliminated. Any difference in white cell count between control and infected blood, which might result in a possible difference in 'background', would not be detected by the present test. The counts can therefore be regarded as an accurate record of  $^3\text{H}$ -hypoxanthine uptake by *Babesia* alone.

The level of parasitaemia, provided it was >5% and not past peak<sup>2</sup>, did not seem to affect the percentage inhibition of  $^3\text{H}$ -hypoxanthine uptake. For example, three trials with amicarbalide against *B. divergens*-infected blood of 12.5%, 18.5% and 24.0% parasitaemia gave the following respective figures for uptake of  $^3\text{H}$ -hypoxanthine, 24.1%, 21.0% and 17.2%.

It is not yet clear how the test works, but a purine salvage pathway involving incorporation of hypoxanthine may be essential for nucleic acid synthesis in *Babesia*<sup>2</sup>, just as in *Plasmodium*<sup>15–17</sup>, in which case any compound which

Table 1 Percentage uptake of  $^3\text{H}$ -hypoxanthine by *Babesia* spp. incubated *in vitro* in the presence of different drugs

| Parasitaemia (%)            | Parasite                                 |                                 |                            |                           |
|-----------------------------|------------------------------------------|---------------------------------|----------------------------|---------------------------|
|                             | <i>B. rodhaini</i><br>18.6 (3)           | <i>B. divergens</i><br>18.3 (3) | <i>B. major</i><br>9.3 (2) | ' <i>B. ovis</i> '<br>5.4 |
| Drug and type               | Uptake of $^3\text{H}$ -hypoxanthine (%) |                                 |                            |                           |
| Purine                      |                                          |                                 |                            |                           |
| Hypoxanthine                | 5.3                                      | 6.5                             | 4.3                        |                           |
| Purine analogues            |                                          |                                 |                            |                           |
| 2-Mercaptopurine            |                                          | 8.7 (2)*                        | 16.2 (2)                   | 7.0                       |
| 6-Mercaptopurine            | 22.9                                     | 42.1 (2)                        | 32.2                       |                           |
| 8-Azaguanine                | 90.1 (2)                                 | 68.1                            | 81.0                       |                           |
| 6-Thioguanine               | 41.1 (2)                                 | 34.9                            | 39.5                       |                           |
| 8-Aza-adenine               | 60.8                                     | 84.1                            |                            |                           |
| Allopurinol                 | 55.7                                     | 34.3                            |                            |                           |
| Pyrimidine analogues        |                                          |                                 |                            |                           |
| Fluorodeoxyuridine          | 82.1                                     | 102.4                           |                            |                           |
| Bromodeoxyuridine           | 73.4                                     | 122.7                           |                            |                           |
| Antifolate                  |                                          |                                 |                            |                           |
| Aminopterin                 | 86.5 (2)                                 | 120.8                           | 72.1                       |                           |
| Babesicides                 |                                          |                                 |                            |                           |
| Trypan blue                 |                                          | 36.7                            | 67.7                       | 90.0                      |
| Quinuronium SO <sub>4</sub> | 28.2                                     | 65.6 (2)                        | 10.2 (2)                   | 39.2                      |
| Imidocarb                   | 14.6                                     | 13.5                            |                            | 32.7                      |
| Diminazene                  | 39.5                                     | 72.1                            | 9.0                        | 16.9                      |
| Amicarbalide                | 10.1 (3)                                 | 20.8 (3)                        | 21.1 (2)                   | 35.7                      |
| Antimalarial                |                                          |                                 |                            |                           |
| Chloroquine SO <sub>4</sub> |                                          | 81.0                            | 113.4                      | 75.4                      |
| Control (no drug)           | 100                                      | 100                             | 100                        | 100                       |

\*Figures followed by (2) or (3) indicate that values are the means of two or three separate readings. Each reading represents the mean from duplicate samples.

Blood from infected and control animals was collected into anticoagulant and washed thrice in PBS (pH 7.2). Aliquots of  $6 \times 10^8$  erythrocytes were placed in 25-cm<sup>2</sup> Falcon flasks containing 10 ml of Eagles' MEM, supplemented with 100 IU ml<sup>-1</sup> of benzyl penicillin, 100 µg ml<sup>-1</sup> streptomycin sulphate, 25 U ml<sup>-1</sup> mycostatin, 50 mM HEPES buffer (pH 7.2) and 10% foetal calf serum. Appropriate drugs were added to cultures at the rate of 20 µg ml<sup>-1</sup>. Cultures were incubated at 37 °C and after 1 h, 2 µCi of  $^3\text{H}$ -hypoxanthine ml<sup>-1</sup> (specific activity 1.8 Ci mmol<sup>-1</sup>) was added to each culture. Cultures were further incubated overnight; erythrocytes were collected after centrifugation at 1,500g and three washings in PBS. Cells were resuspended in 5 ml of PBS; 1-ml samples were transferred to 5 ml of 5% ice-cold TCA and duplicate 1-ml aliquots of this suspension were passed through separate 2.5-cm diameter 1.2-µm Millipore filters. Filter pads were washed and dried and then placed in scintillation vials containing 10 ml of toluene-based scintillation fluid. Counts per minute were recorded after 10 min of counting in a liquid scintillation analyser at 8 °C. Uptake of  $^3\text{H}$ -hypoxanthine by parasites was recorded after counts were corrected to eliminate background and uptake by non-infected cells.

**Table 2** Percentage uptake of  $^3\text{H}$ -hypoxanthine by normal and Diminazene-resistant<sup>19</sup> strains of *B. rodhaini* incubated *in vitro* in the presence of different babesicidal drugs

| Strain of <i>B. rodhaini</i> | Control | Amicarbalide | Drug<br>Quinuronium SO <sub>2</sub> | Imidocarb | Diminazene |
|------------------------------|---------|--------------|-------------------------------------|-----------|------------|
| Normal                       | 100     | 17.3         | 28.2                                | 14.6      | 39.5       |
| Diminazene-resistant         | 100     | 11.6         | 32.9                                | 5.0       | 77.9       |

Drug concentrations and experimental methods as for Table 1.

inhibits parasite multiplication, and thus nucleic acid synthesis either directly or indirectly, will result in a reduction of hypoxanthine uptake. The test may therefore be measuring the degree of drug-induced inhibition of nucleic acid synthesis. Although the mode of action needs to be determined, the test seems to be reliable, simple, quick and cheap; it does not involve the use of large numbers of animals; it can be applied to any species of *Babesia*; and the drugs do not require previous toxicity testing. Before *in vivo* tests, active compounds can be further screened at different concentrations *in vitro* and structure-activity relationships<sup>18</sup> can be quickly evaluated.

Preliminary tests with a Diminazene-resistant strain of *B. rodhaini*<sup>19</sup> suggest that drug resistance patterns may also be detected by the inhibition of  $^3\text{H}$ -hypoxanthine uptake. The results shown in Table 2 agree with *in vivo* tests<sup>19</sup> which have shown that a Diminazene-resistant strain was also resistant to Quinuronium sulphate but less resistant to Amicarbalide and Imidocarb.

*In vitro* tests for the detection of antimalarial drug activity, based on the inhibition of  $^{32}\text{P}$  and  $^3\text{H}$ -adenosine uptake by *Plasmodium*-infected blood, have been described<sup>20,21</sup> but never widely adopted, possibly because of their complexity or lack of versatility. The test described here does not seem to be subject to these constraints and, since many malaria parasites are known to incorporate hypoxanthine<sup>15,16</sup> readily, the test could also be considered for the primary screening of antimalarial drugs.

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## Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats

ONE experimental approach to male contraception has been the systemic administration of androgenic steroids, but treatment has failed sometimes to induce azoospermia<sup>1-4</sup>. To overcome this difficulty, androgen-progestin formulations have been tested, but they did not inhibit spermatogenesis completely in all treated men<sup>5-8</sup>. We thought that this variable response was probably due to the relatively weak anti-gonadotropic activity of both androgens and progestins and the difficulty of controlling precisely the dose of steroids administered. We reasoned that azoospermia might be achieved in every male if steroids were administered continuously and at relatively constant rates and if progestins were replaced by a more potent inhibitor of pituitary gonadotropin secretion. We describe here the results of a test of the efficacy of testosterone-oestradiol formulations administered to adult male rats by means of a subdermal sustained release device. Oestradiol was used because, like testosterone, it is a naturally occurring steroid secreted by the mammalian testis<sup>9-13</sup> and because it is a potent inhibitor of pituitary gonadotropin secretion<sup>14-16</sup>.

Ninety adult male Sprague-Dawley rats (225-260 g) were assigned randomly to one of 15 treatment groups. Subdermal implants of Silastic of different sizes, containing either testosterone or oestradiol-17 $\beta$  were used to administer steroid. These sustained release devices were constructed from Silastic (polydimethylsiloxane) medical grade tubing because studies in our laboratory have established that such devices release hormonal steroids at relatively constant rates for a long time<sup>17,18</sup>. Three groups of 30 rats each were given either no oestradiol-17 $\beta$  or oestradiol-17 $\beta$ -filled Silastic implants measuring 0.1 cm or 0.3 cm (release rate for oestradiol was approximately 0.1  $\mu\text{g cm}^{-1} \text{d}^{-1}$ ). The 30 rats in each group were further subdivided into five groups of six rats each, receiving either no testosterone or testosterone-filled Silastic implants measuring 1.0, 2.5, 4.0 or 6.0 cm (release rate for testosterone was approximately 30  $\mu\text{g cm}^{-1} \text{d}^{-1}$ ). The implants were prepared by filling Silastic tubing (Dow Corning No. 602-305) with steroid and implanting it subdermally as before<sup>17-18</sup>. Three months later the rats were weighed and decapitated, trunk blood was collected and serum was prepared for radioimmunoassay of oestradiol<sup>19</sup>, testosterone<sup>20</sup> and luteinising hormone (LH)<sup>21</sup>. The seminal vesicles and ventral prostate were dissected, freed of extraneous tissue and weighed; the fluid was expressed from seminal vesicles before weighing. Spermatids and spermatozoa were counted haemocytometrically.

**Table 1** Average number of spermatozoa and spermatids in adult male rats with subdermal implants containing testosterone or oestradiol

| Oestradiol implant length (cm) | Testosterone implant length (cm) |             |             |            |             |
|--------------------------------|----------------------------------|-------------|-------------|------------|-------------|
|                                | 0                                | 1           | 2.5         | 4          | 6           |
| 0                              | 234 $\pm$ 12                     | 193 $\pm$ 9 | 61 $\pm$ 39 | 17 $\pm$ 6 | 80 $\pm$ 19 |
| 0.1                            | 194 $\pm$ 18                     | 38 $\pm$ 26 | <1*         | 48 $\pm$ 2 | 85 $\pm$ 7  |
| 0.3                            | 205 $\pm$ 15                     | <1          | <1          | 3 $\pm$ 1  | 43 $\pm$ 11 |

Values represent the mean  $\pm$  s.e.m. of six animals.

\*Indistinguishable from zero.



**Table 2** Effect of testosterone and oestradiol in subdermal implants on serum LH, testosterone and oestradiol and on the weight of seminal vesicles and ventral prostate in adult male rats

|                                     | Treatment    |                     |                   |                                            |
|-------------------------------------|--------------|---------------------|-------------------|--------------------------------------------|
|                                     | Control      | 2.5 cm Testosterone | 0.1 cm Oestradiol | 2.5 cm Testosterone plus 0.1 cm oestradiol |
| LH (mg ml <sup>-1</sup> )*          | 8.0 ± 1.0    | 2.9 ± 0.3           | 2.8 ± 0.4         | ND                                         |
| Testosterone (ng ml <sup>-1</sup> ) | 2.3 ± 0.5    | 3.0 ± 0.6           | 0.9 ± 0.5         | 2.2 ± 0.2                                  |
| Oestradiol (pg ml <sup>-1</sup> )   | 45.8 ± 11.0  | 47.5 ± 11.0         | 46.2 ± 3.0        | 42.7 ± 4.0                                 |
| Paired seminal vesicle weight (mg)  | 454.0 ± 8.0  | 464.0 ± 26.0        | 258.0 ± 28.0      | 451.0 ± 21.0                               |
| Ventral prostate weight (mg)        | 577.0 ± 62.0 | 686.0 ± 57.0        | 358.0 ± 36.0      | 678.0 ± 34.0                               |

\*Values are expressed in terms of ng equiv of NIAMD-rat-LH-RP-1 per ml of blood serum. The rat LH standard has a relative potency of 0.03 times the NIH standard (NIH-LH-S1) in the ovarian ascorbic acid depletion assay.

Each value represents the mean ± s.e.m. of the mean of six animals.

ND. Not detectable.

from homogenates of testes from each animal<sup>22</sup>. Testicular perfusions were done as described earlier<sup>23-24</sup>.

Testosterone administered in the absence of oestradiol (Table 1) caused the expected biphasic changes in the combined number of spermatozoa and/or spermatids per testis<sup>25</sup>. Numbers of spermatids and spermatozoa did not reach zero in any of the five treatment groups: this differs from the result reported by Berndtson *et al.*<sup>25</sup>. The difference probably resulted because Berndtson *et al.* quantified spermatogenesis by counting germ cells in histological sections of testes<sup>25</sup>, whereas we used a haemocytometer to count nuclei of spermatids and spermatozoa in a testicular homogenate. No significant alterations in spermatogenesis were apparent in rats receiving oestradiol without testosterone (Table 1). Surprisingly, testosterone and oestradiol doses (for example, 1.0 cm testosterone plus 0.1 cm oestradiol) that failed significantly to inhibit spermatogenesis when administered alone, markedly inhibited spermatogenesis when given simultaneously (Table 1). This interaction is apparently synergistic (supra-additive) because increasing the dose of either steroid by more than two-fold did not result in the major suppression of spermatogenesis obtained with the combination of testosterone and oestradiol.

The testes of each animal, treated with several of the testosterone-oestradiol combinations, were devoid of condensed spermatids and spermatozoa. At the lower dose of oestradiol-17β (0.1-cm implant), azoospermia was first obtained with a 2.5-cm Silastic implant of testosterone (Table 1). Consequently, further measurements were completed on animals receiving this steroid formulation.

The results in Table 2 suggest that the 2.5-cm testosterone-0.1 cm oestradiol treatment inhibited spermatogenesis indirectly by suppressing release of immunoreactive LH. We cannot, however, rule out a direct effect of oestradiol on testosterone secretion because Chowdhury *et al.*<sup>26</sup> suggested that oestrogen inhibits testicular steroidogenesis. Serum concentrations of testosterone and oestradiol were similar to control values in rats receiving the combination treatment (Table 2). Serum testosterone concentration reflected the free biologically active hormone, for the accessory sex organ weights of these rats were not significantly different from those of control rats.

These results raise two important questions about the mechanism by which testosterone-oestradiol combinations induced azoospermia, because the circulating levels of each steroid remained within the range typically noted in the sera of untreated adult male rats. First, why were concentrations of peripheral blood serum testosterone and oestradiol not increased? The probable answer is that testes of rats receiving the 2.5-cm testosterone-0.1-cm oestradiol treatment failed to produce testosterone. Testosterone secretion by *in vitro* perfused testes from control rats and rats with the 2.5-cm testosterone-0.1-cm oestradiol implants was 5.0 ± s.e.m. 0.4 (n=6) and 0.1 ± s.e.m. 0.03 (n=6) μg h<sup>-1</sup>. Thus it seems reasonable to conclude that the implant was the major source of testosterone in the peripheral blood of the aspermato-genic rats. The steroids thus released replaced those usually

derived directly from the testis or indirectly from peripheral conversion of testicular steroids. Presumably testosterone from the implants was sufficient to maintain peripheral androgen-dependent functions but insufficient to maintain the high intra-testicular concentration of testosterone<sup>25,27</sup> required for spermatogenesis. The second question is: how did normal circulating titres of testosterone and oestradiol completely suppress circulating levels of immunoreactive LH? Probably because, in contrast to the episodic fluctuations in testosterone titres seen in normal males<sup>28</sup>, implants release steroids at relatively constant rates<sup>17,29,30</sup>. Thus it seems that sustained release of testosterone provides a more effective signal to androgen-dependent target tissues than the episodic fluctuations characteristic of intact animals<sup>25,31</sup>.

Thus azoospermia can be induced in adult male rats with the appropriate testosterone-oestradiol formulation administered from a subdermal sustained release device. Preliminary results from our laboratory suggest that these azoospermic rats are sexually active but, predictably, infertile. Taken together, these results indicate that androgen-oestrogen combinations are an effective male contraceptive formulation in the rat, and thus the suggestion of Briggs and Briggs<sup>32</sup> to test such combinations in the human male should be taken up.

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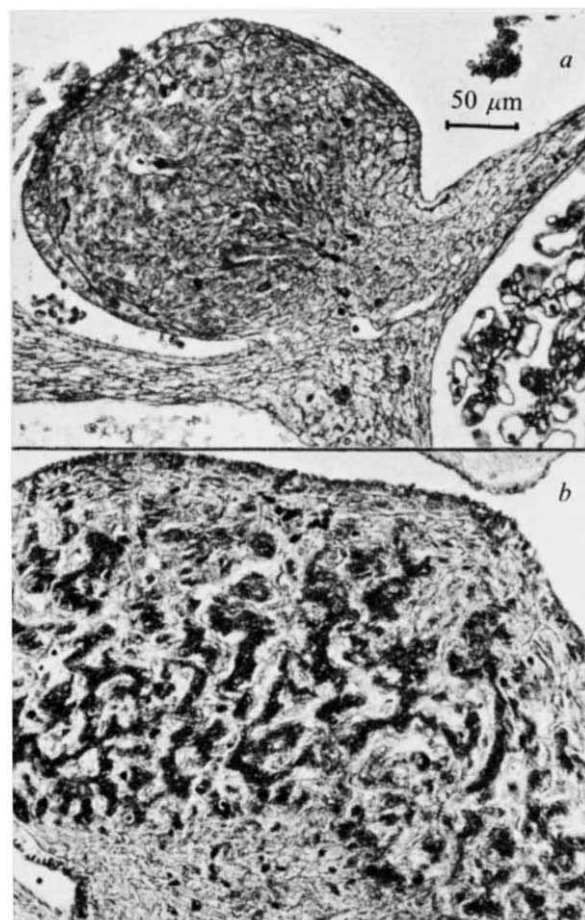
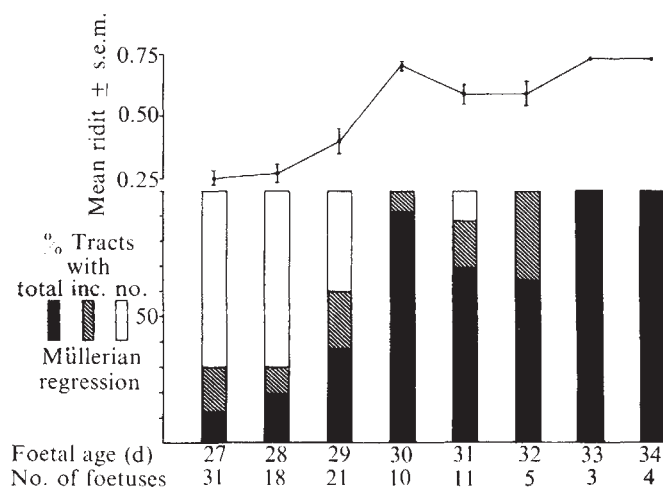
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## Anti-Müllerian hormone is a functional marker of foetal Sertoli cells

REGRESSION of Müllerian ducts in male foetuses is mediated by a testicular factor distinct from testosterone<sup>1</sup>. This anti-Müllerian hormone (AMH), a protein which has been partially purified<sup>2</sup>, is synthesised by foetal Sertoli cells<sup>3</sup>. In mammals the first stage of testicular differentiation involves the arrangement of sustentacular or Sertoli cells around the gonocytes and their encasement in testicular cords. Leydig cells appear later in the interstitium and their differentiation is correlated with the onset of steroidogenesis. We thought that there might also be a correlation between the initiation of testicular anti-Müllerian activity and the differentiation of Sertoli cells. Little work has been done on anti-Müllerian activity of the early foetal testis. During the initial stages of testicular organogenesis in the rat<sup>4</sup> and the guinea pig<sup>5</sup>, AMH production is low, but the rapid pace of sex differentiation in most rodents hampers sequential study. Using sows (*Sus scrofa*), we have avoided this problem, and confirmed that the correlation exists.

Gestational age is known with great accuracy for sows of the large-white breed, bred at the INRA Zootechnical Centre. Foetuses were tested for testicular anti-Müllerian activity at between 27 and 34 d of gestation. Sex was determined by cytogenetic examination of hepatic cells<sup>6</sup>

**Fig. 1** Chronological development of anti-Müllerian activity of the foetal pig testis. See text for details. Inc., incomplete.



**Fig. 2** Histological aspect of the pig foetal testis studied by the silver impregnation method of Gomori<sup>13</sup>. *a*, 27 d: undifferentiated gonad; testicular cords are not clearly outlined. The mesonephros is on the right. *b*, 29 d: testicular organogenesis is complete; both the tunica albuginea and testicular cords are well differentiated.

from foetuses up to 30 d and by inspection of the genital tract in older ones. Testicular tissue was explanted on an agar-coated grid placed in a Petri dish filled with Ham's F12 medium, and the anti-Müllerian activity of between two and five explants was tested promptly by association with 14.5-d-old gonadless rat reproductive tracts<sup>7</sup>. In some 27 and 29-d-old pig foetuses one testis was cultured alone for 3 d before testing. The test was terminated after 3 d by fixation and the degree of regression of the Müllerian ducts of individual rat reproductive tracts exposed to testicular explants was scored as before<sup>8</sup>.

Figure 1 shows that anti-Müllerian activity increased steadily from negligible levels at 27 d to maximal values at 33 d. The ridit method<sup>9,10</sup> of transformation of a non-parametric variable revealed a highly significant correlation ( $r = 0.59$ ,  $P < 0.01$ ) between anti-Müllerian activity and foetal age. We also found that anti-Müllerian activity could be acquired *in vitro*, though at a slower rate. Testes from 27-d-old pig foetuses after culture for 3 d had a significantly higher mean anti-Müllerian activity ( $P < 10^{-1}$  by Student's paired *t* test) than the contralateral testes but lower activity ( $P < 10^{-7}$  by Student's *t* test) than testes explanted at 30 d of gestation. The same tests performed on day 29 did not yield significant results (Table 1), probably because the basal level of anti-Müllerian activity was already high.

Gonadal sex is recognisable by electron microscopy as early as day 26 in the pig foetus<sup>11</sup>. Using a light microscope, however, we were unable reliably to identify testicular organogenesis before day 29. Even then, primitive testicular cords, two to three cells wide, as described by



**Table 1** *In vitro* development of anti-Müllerian activity of foetal pig testis

| Age (d) | No. of pig foetuses | No. of tracts associated | Mean ridit $\pm$ s.e.m. | Comparison with preceding group |
|---------|---------------------|--------------------------|-------------------------|---------------------------------|
| 27      | 25                  | 25                       | 0.246 $\pm$ 0.030       |                                 |
| 27 + 3* | 25                  | 25                       | 0.440 $\pm$ 0.048       | $P < 10^{-3}\dagger$            |
| 30      | 10                  | 35                       | 0.697 $\pm$ 0.017       | $P < 10^{-7} (\ddagger)$        |
| 29      | 13                  | 13                       | 0.406 $\pm$ 0.048       |                                 |
| 29 + 3* | 13                  | 13                       | 0.626 $\pm$ 0.055       | NS†                             |
| 32      | 5                   | 14                       | 0.588 $\pm$ 0.049       | NS‡                             |

The mean ridit<sup>9,10</sup> expresses the probability that a rat reproductive tract selected from those exposed to pig testicular tissue from a given age group will have a greater degree of Müllerian regression than one selected at random from the reference population (here, all 246 tracts used in the study). NS, Not significant.

\*Days in culture before test for anti-Müllerian activity.

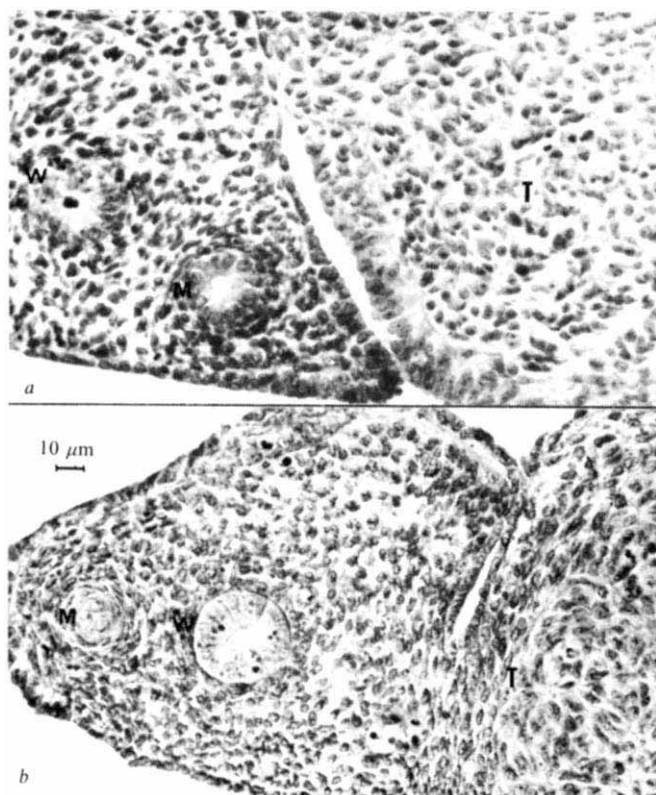
†By Student's paired bilateral *t* test.

‡By Student's bilateral *t* test.

Moon and Hardy<sup>12</sup>, were visible only after treatment with Gomori's silver impregnation<sup>13</sup> (Fig. 2). At day 29, also, testicular anti-Müllerian activity becomes clearly demonstrable (Fig. 3). It seems therefore that cellular rearrangements characteristic of seminiferous tubule organisation must be completed before the activity of early Sertoli cells can be demonstrated physiologically. Similarly, Leydig cells differentiate in the interstitium at approximately day 31 (ref. 12), but peak testosterone concentrations are reached only by day 37 in the testis<sup>11</sup> and day 55 in the plasma<sup>15</sup>.

Testicular anti-Müllerian activity wanes and eventually

**Fig. 3** Anti-Müllerian activity of foetal pig testis, cocultured for 3 d with 14.5-d-old gonadless rat reproductive tract. *a*, 27 d at explantation: slight inhibition of the Müllerian duct is shown by the formation of a peri-Müllerian fibroblastic ring. *b*, 29 d at explantation: the Müllerian epithelium has almost completely disappeared, replaced by a fibrous whorl. M, Müllerian duct; W, Wolffian duct; T, pig foetal testis. Periodic acid Schiff stain was used.



vanishes after birth (see ref. 2 for review). This led Josso<sup>18</sup> to conclude that AMH is a foetoprotein, that is no longer synthesised after the peri-natal period. In the light of the subsequent demonstration of the Sertoli-cell origin of AMH<sup>3</sup>, however, an alternative possibility should be considered: formation of the blood-testis barrier could prevent detection of AMH by inhibiting its release from testicular explants. After the formation of occluding junctions between adjacent Sertoli cells, the products of these cells such as inhibin or androgen-binding protein, accumulate in seminal fluid and epididymis<sup>17,18</sup>, where a search for AMH might be rewarding for the understanding of the relationship between the function of the post-natal Sertoli cells and anti-Müllerian activity.

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## ***In vitro* duplication and 'cure' of haemopoietic defects in genetically anaemic mice**

SEVERAL types of congenital, genetically determined macrocytic anaemias have been described in mice<sup>1–4</sup>. These models offer a means of analysing the effects of mutations at defined loci on the ability of haemopoietic tissue to support stem cell proliferation and differentiation and allow studies on intrinsic defects in differentiation capacity of stem cells. But, analysis of these mutations and their implications in relation to pluripotential stem cell control have been hampered by lack of techniques, particularly of *in vitro* systems, for further defining regulatory interactions between stem cells and the haemopoietic inductive microenvironment. Here we report that the haemopoietic defects seen in W and Steel (Sl) mice have been successfully reproduced *in vitro*.

The pleiotropic effects of mutations at the W and Steel (Sl) loci include macrocytic anaemia, extreme radiosensitivity, sterility and defective pigmentation<sup>1</sup>. The lesions in W mice are presumed to be due to intrinsic defects in haemopoietic stem cells, primordial germ cells and melanoblast precursors<sup>1–4</sup>, whereas in Sl mice the defect resides within the environment into which these cells migrate, that is, the haemopoietic microenvironment, the gonads and skin<sup>1,2,4,5</sup>. Support for this view is provided by the observation that pluripotential stem cells detected by the spleen



**Table 1** Production of CFU-c and differentiating haemopoietic cells in continuous cultures of  $W/W^v$ ,  $Sl/Sl^d$  and control bone marrow combinations

| Genotype of adherent cells | Genotype of marrow cells added | Total non-adherent cells per culture ( $\times 10^{-5}$ ) |      |      |      |      |      | Total CFU-c per culture |        |        |        |       |        |
|----------------------------|--------------------------------|-----------------------------------------------------------|------|------|------|------|------|-------------------------|--------|--------|--------|-------|--------|
|                            |                                | Weeks cultured*                                           |      |      |      |      |      | Weeks cultured          |        |        |        |       |        |
|                            |                                | 1                                                         | 2    | 3    | 4    | 5    | 6    | 1                       | 2      | 3      | 4      | 5     | 6      |
| $Sl(+/+)$                  | $Sl(+/+)$                      | 25.0                                                      | 24.0 | 10.0 | 70.0 | 8.6  | 14.0 | 16,000                  | 10,600 | 10,100 | 41,700 | 2,300 | 12,200 |
| $Sl/Sl^d$                  | $Sl/Sl^d$                      | 18.0                                                      | 14.0 | 2.4  | 1.6  | 6.0  | 9.0  | 21,900                  | 8,300  | 2,300  | 540    | 1,300 | 90     |
| $W(+/+)$                   | $W(+/+)$                       | 52.0                                                      | 24.0 | 30.0 | 28.0 | ND   | ND   | 32,000                  | 15,200 | 22,800 | 23,000 | ND    | ND     |
| $W/W^v$                    | $W/W^v$                        | 20.0                                                      | 9.4  | 4.0  | 3.2  | ND   | ND   | 2,500                   | 430    | 190    | 260    | ND    | ND     |
| $Sl/Sl^d$                  | $W/W^v$                        | 5.6                                                       | 9.0  | 6.8  | 1.2  | 4.0  | ND   | 150                     | 0      | 0      | 0      | 0     | ND     |
| $W/W^v$                    | $Sl/Sl^d$                      | 40.0                                                      | 7.0  | 20.0 | 58.0 | 30.0 | ND   | 46,200                  | 4,500  | 11,900 | 13,800 | 7,500 | ND     |

ND, Not determined.

\*Weeks from time of addition of the second marrow.

colony assay (colony-forming units, CFUs) are greatly reduced or absent in  $W$  anaemic mice but are present in normal numbers in  $Sl$  mice<sup>1</sup>. Furthermore,  $W$  mice, both irradiated or unirradiated, support spleen colony formation after inoculation of bone marrow from non-anaemic litter mates or from  $Sl$  mice, whereas spleen colony formation does not occur in irradiated  $Sl$  mice after normal marrow inoculation<sup>1,5</sup>. The haematological defects of  $W$  mice can, therefore, be cured by an inoculum of marrow cells from  $Sl$  mice and the macrocytic anaemia of  $Sl$  mice can be reversed after grafting of neonatal spleen or whole bone from  $W$  anaemic mice as a source of non-defective haemopoietic microenvironment. A culture system has been developed in which the proliferation of murine pluripotent stem cells (CFU-s), committed granulocyte-macrophage stem cells (CFU-c) and differentiating granulocytic cells can be maintained *in vitro* for several months<sup>6-8</sup>. In this system mouse bone marrow-derived adherent cell cultures established for 3 weeks are re-inoculated with freshly isolated samples of syngeneic, allogeneic or semi-allogeneic bone marrow cells. The latter subsequently undergo proliferation for several months. This proliferation, differentiation and maturation of haemopoietic cells is dependent on the formation of a bone marrow-derived adherent population comprised of phagocytic mononuclear cells, 'endothelial' cells and giant fat-containing cells which apparently provide an *in vitro* microenvironment necessary for pluripotent stem cell renewal and differentiation<sup>7</sup>. Within this adherent layer extensive cellular interactions occur reminiscent of the intimacy of inductive cell interactions seen in embryogenesis. We have used this system to further explore the haemopoietic defects in  $W$  and  $Sl$  mice. To establish the cultures, the contents of a single femur from 10-14-week-old  $WBB6F_1-W/W^v$  ( $W/W^v$ ),  $WCB6F_1-Sl/Sl^d$  ( $Sl/Sl^d$ ) or their normal litter mates  $W(+/+)$ ,  $Sl(+/+)$ , (Jackson Laboratories) were flushed into glass culture bottles containing 10 ml of Fisher's medium (Gibco), supplemented with antibiotics and 25% horse serum (Flow Labs), as described previously<sup>6-8</sup>. The cultures were incubated at 33 °C (a temperature shown to facilitate the development of the adherent layer of cells and subsequent growth of stem cells) in an atmosphere of 5%  $CO_2$  in air. At weekly intervals the cultures were 'fed' by removal of half the growth medium and addition of an equal volume of fresh medium. Over a period of 2-3 weeks an adherent layer of cells became established and at 3 weeks all the growth medium and suspension cells were removed and the adherent layer re-fed with 10 ml of growth medium containing  $10^7$  freshly isolated bone marrow cells. The cultures were incubated at 33 °C and at weekly intervals were fed by removal of half the growth medium suspension cells and addition of fresh medium. The suspension cells removed at each weekly depopulation were assayed for granulocyte-macrophage progenitor cell (CFU-c) growth in soft agar using conditioned medium from a murine myelomonocytic leukaemic cell line (WEHI-3) as a source of colony stimulating factor.

Pooled cells from at least six cultures were used for each time point.

As shown in Table 1, extensive cell production was observed over a 6-week period in control cultures of non-anaemic  $WBB6F_1$  ( $W(+/+)$ ) or  $WCB6F_1$  ( $Sl(+/+)$ ) bone marrow. Production of CFU-c was likewise maintained at a high level throughout the period of culture. In contrast, cultures of  $Sl/Sl^d$  marrow or of  $W/W^v$  marrow showed considerably reduced production of both haemopoietic cells and CFU-c to as little as 1-3% of litter mate control cultures by 4 weeks. The morphology of the cultured cells showed a preponderance of differentiating granulocytic elements (40-75%) in the first 4-5 weeks of culture with an increasing proportion of phagocytic mononuclear cells at later stages. Transition to a macrophage morphology was observed earlier in  $W/W^v$  and  $Sl/Sl^d$  cultures than in the control cultures.

Allogeneic cultures were also initiated using  $Sl/Sl^d$  marrow to establish an adherent layer which was subsequently fed with  $W/W^v$  bone marrow. The additive effects of the  $Sl$  environmental defect and the  $W$  stem cell defect were apparent in this combination since cell production in culture was again markedly reduced relative to the control cultures and CFU-c were undetectable by the second week of culture. In addition, granulopoiesis in these cultures was no longer evident after the first week and thereafter cell proliferation was exclusively macrophage. The reverse combination using  $W/W^v$  to initiate the adherent population and subsequent feeding with  $Sl/Sl^d$  marrow resulted in a sustained high level of production of both differentiating haemopoietic cells and CFU-c throughout the period of observation.

While we have not as yet systematically monitored CFU-s in these cultures, the striking defect in generation of CFU-c and of differentiating haemopoietic cells seen in the  $W/W^v$ ,  $Sl/Sl^d$  and  $Sl/Sl^d-W/W^v$  cultures clearly points to an underlying defect at the level of interaction of the adherent marrow cells with the pluripotent stem cell.

But, no qualitative or quantitative defect was seen in the capacity of  $Sl/Sl^d$  marrow to generate an adherent layer and the defect may lie at the level of cellular interactions. Ultrastructural analysis of the *in vitro* microenvironment is being performed. The duplication of the  $W$  and  $Sl$  defect *in vitro* and our ability to 'cure' the defect by appropriate combinations of adherent cells and stem cells should permit further insight into genetically determined haemopoietic disorders and inductive cell interactions associated with pluripotent stem cell self renewal and differentiation.

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## HLA 'help' for human B2-microglobulin across species barriers

HLA antigens comprise products of what is commonly considered to be one of the most polymorphic systems presently known, occurring on chromosome 6. The HLA epitope is present only once<sup>1</sup> on a glycoprotein of molecular weight 45,000. In its naturally occurring form it is tightly, and probably obligatorily, but non-covalently bound to B2-microglobulin, (B2M), a protein of molecular weight 11,600 (ref. 2), determined by chromosome 15. B2M has substantial sequence homology with the C<sub>H</sub>3 domain of IgG (ref. 3), and seems to be non-polymorphic. Precisely analogous associations occur between several other MHC antigens in rodents and fowl, and the equivalents of B2M in those species. This leads to the question as to why products of probably the most polymorphic systems should be so strongly associated with a non-polymorphic protein. In this paper we confirm that human B2M is immunogenic in phylogenetically distant species when the molecule is presented alone. When the species barrier is close, immunogenicity declines drastically as, for example, between man and other primate. In contradistinction, however, when B2M is presented bound to the HLA alloantigen-bearing chain, as occurs naturally, the complex is exquisitely immunogenic with respect to both B2M and the alloantigenic determinant if the species are closely related, and may decline as the species barrier widens. This is interpreted in terms of a modified 'intramolecular' help effect across a phylogenetically closely related barrier.

Free human B2M is clearly immunogenic in several

species at doses of 25–100 µg. As might be expected, antibody titres in chickens are higher than in mammals, reflecting a wider species barrier. Hyperimmune rabbit antisera to the unseparated proteins in human serum have also been examined (last line, Table 1). Although B2M was detected in the immunising inoculum at levels of 1–2 µg ml<sup>-1</sup>, no anti-B2M antibodies were found in the resultant antisera, confirming that, at this dose, B2M is essentially non-immunogenic.

Much smaller doses (0.1–0.2 µg) of B2M bound to HLA antigen are highly immunogenic in rabbits and anti-B2M antibodies rise to levels essentially similar to those found with much higher doses of B2M used alone. As expected, the sera are cytotoxic for peripheral human lymphocytes. Furthermore, the immunogen used did not inhibit the lysis of B cells by a rabbit anti-human B cell-specific antiserum<sup>4</sup>, thereby suggesting the absence of human Ia antigens in the preparations. (Contamination with Ia antigens is in any case probably ruled out by the anion exchange and gel filtration purification procedures used.)

Absorption of the rabbit anti-HLA sera with human erythrocytes (which are lacking in B2M and HLA antigens), did not reduce the lytic titre by much. Absorption with platelets or crude human spleen membranes negative for the immunising HLA specificity were, however, much more effective in removing lympholytic antibodies. Nevertheless, drastic absorption (>95%) was necessary before moderate HLA specificity (SR>5) was revealed. Antibodies to B2M contributed substantially to the original cytotoxicity. This is most clearly shown by passing antiserum through a B2M affinity column, when 90–95% of cytotoxicity was removed; neither was there a notable increase in HLA allospecificity following such treatment.

In spite of this low level of allospecific globulin, such lytic rabbit sera are substantially inhibited by HLA antigens, thereby confirming earlier work<sup>5</sup> in which nonspecific cytotoxic xenoantisera prepared against H2 or HLA antigens in soluble or cellular form were inhibited only by those areas of a gel filtration chromatogram (of crude soluble membrane proteins) containing mouse H-2D and H-2K alloantigens or human HLA-A, B, and C alloantigens respectively. Rabbits are known to raise antibodies to at least one common

Table 1 Sensitisation of non-primate species to human B2M

| Species*   | Injected protein per dose (µg) | B2M form                          | Adjuvant† | Anti-B2M‡ titre | Human lymphocytotoxins 50% Cytotoxic titre§ | SR¶       |
|------------|--------------------------------|-----------------------------------|-----------|-----------------|---------------------------------------------|-----------|
| Chicken    | 50                             | Free                              | C,A       | 5,000           | 500 (chicken complement)                    | 1.0       |
| Rat        | 25                             | Free                              | C,A       | 648 ± 233       | —                                           | —         |
| Guinea pig | 50                             | Free                              | C,A       | 358 ± 275       | —                                           | —         |
| Rabbit     | 50–100                         | Free                              | C,A       | 1,107 ± 407     | 60 ± 16                                     | 1.0       |
| Rabbit     | 0.5–1.0**                      | Papain solubilised HLA antigens†† | I,A       | 838 ± 435       | 889 ± 265                                   | 1.2 ± 0.2 |
| Rabbit     | Human serum proteins           | Free                              | C,A       | 0               | —                                           | —         |

\*Groups of four or five animals were usually used. When free B2M was used as antigen, progressively lower doses gave correspondingly lower anti-B2M responses. No detectable response was produced in guinea pigs, chickens, or rabbits with repeated injections of less than 5.0 µg B2M in adjuvant.

†C, Complete Freund's adjuvant; A — alum precipitate; I — incomplete Freund's adjuvant.

‡5 µl of <sup>125</sup>I-B2M solution was added to 8 µl volumes of fivefold dilutions of antiserum in diluent. After incubating at room temperature for 1 h, 0.180 ml 19% Na<sub>2</sub>SO<sub>4</sub> solution was added followed by further incubation at room temperature for 1 h. After centrifugation at 1,000g for 5 min, 50% of the supernatant was sampled. Results were plotted as the percentage radionuclide remaining in solution against antiserum dilution in the reaction volume. The titre of an antiserum is the dilution at which 50% of the nuclide is precipitated. Control samples contained diluent in place of antiserum and results were corrected according to the titration value given by a reference chicken anti-human B2M serum.

§Estimated in microtitre plates using 25-µl volumes of lymphocytes, antiserum dilutions, and rabbit complement, and a Trypan blue indication of cell death.

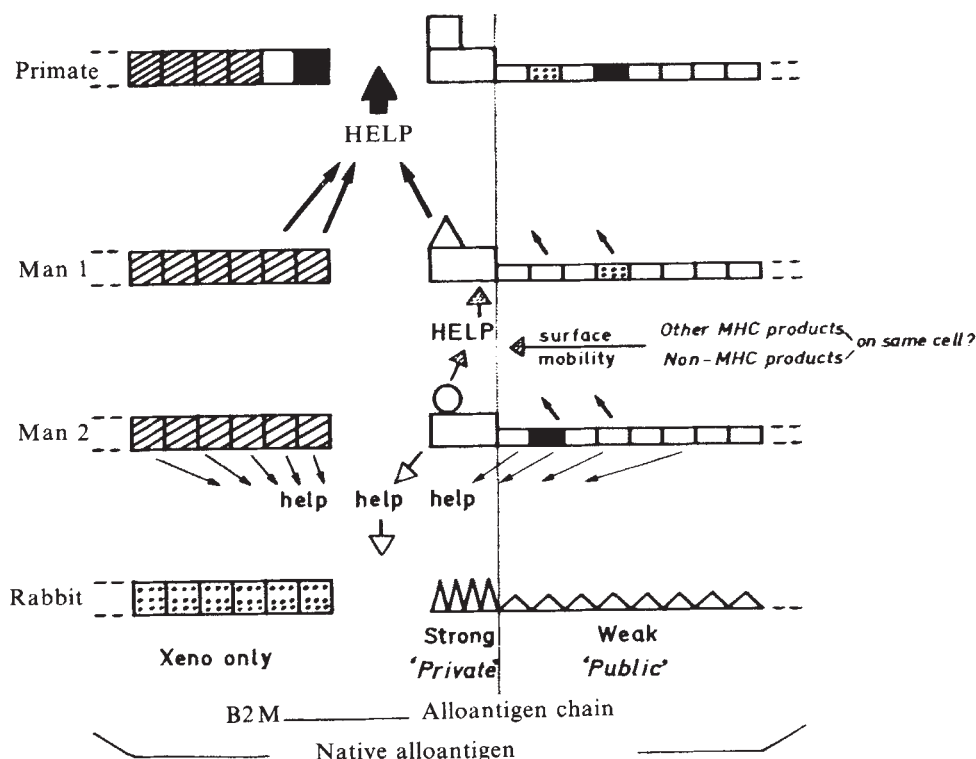
¶SR, Specificity ratio; the ratio of titre with cells positive or negative for an HLA specificity, based on analysis of a population of 20 cell types covering the relevant specificities.

||Values obtained after at least four booster injections in the case of free B2M, but after only two boosts of HLA antigens.

\*\*The protein content of HLA antigen is based on an assumed final purity of 10%, validated by radioimmunoassay of B2M content.

††Specifically purified and separated HLA-A1, A2, B8, and B12 papain-solubilised antigens were used in a total of six rabbits.

**Fig. 1** Associative recognition in alloantigens. 'Help' or associative recognition within a species (man 1-man 2) for antibody production to B2M-associated alloantigens occurs in a primary response only when other MHC region products are present at the same time. It seems likely that these products are required to be on the same cell. If spatial relationships are vital in the presentation to recognition units on T cells, then surface mobility, that is, a living membrane will be an equally vital feature of the allogeneic stimulus. These demands disappear when epitopic differences are revealed in the B2M component of alloantigens as in xenogeneic but phylogenetically close sensitisations (man-primate). Inherent in this argument is the assumption that the remainder of the alloantigenic glycoprotein is poorly immunogenic for whatever reason, and will remain essentially as weak between related species as it does within species. When the barrier is wide, however (man-rabbit), even this restriction disappears, and enough differences occur between the respective species alloantigens that multiple dual recognitions can arise at various places on the sensitising molecule.



species determinant on the alloantigen-bearing chain of all HLA antigens<sup>6</sup>.

Thus antisera prepared in rabbits, even to purified HLA antigens, are not likely to produce reagents of great value in revealing HLA allospecificity on human lymphocyte targets using lytic assays. The rabbit might be expected to differ from man at several places other than the HLA epitope on the HLA chain, as well as at several places on B2M, so allospecific globulins would comprise only a minor portion of the total antibody formed against the whole alloantigen chain with its attendant B2M. The cytotoxins directed against non-allospecific determinants, both on the HLA chain and on human B2M, can be removed by appropriate absorption with specific immunogen-negative tissue. Such an absorbed antiserum, albeit of low cytotoxic titre, has already been extensively examined ( $\chi^2$  with respect to HLA-A1=26.9, cited in ref. 7). What is interesting is that substantially augmented immunogenicity to human B2M is observed when this protein is associated with HLA alloantigen and presented across a species barrier.

The results obtained by immunisation of *Macaca irus* monkeys were quite different. After extensive immunisation with free B2M at doses similar to those which readily produced antibodies in several other non-primate species, much lower humoral immunity was observed with this primate species. Not only were antibody titres lower, but a response was only obtained after six courses of immunisation, compared with two in non-primate species, and the titre has not increased on further immunisation.

Purified HLA substances have previously been shown to be highly immunogenic across this close barrier, producing very high titres of lymphocytotoxic antibodies with a few micrograms of material<sup>8,9</sup>. The absorbed sera (SR  $13.8 \pm 5.6$ ) showed excellent accord with well defined single HLA specificities in population studies. Both the specificity of the unabsorbed sera (SR  $4.8 \pm 1.1$ ), and the yield following absorption indicated that anti-HLA globulins formed the majority of the total lympholytic antibody, in contrast to the results obtained with rabbits and shown earlier.

The results of Table 2 indicate that the limited non-

specificity of the primate antisera is largely attributable to their anti B2M content. First, very high titres of anti B2M antibodies were found in the radioimmunoassay. These levels are often substantially higher than those found in non-primate antisera produced by immunisation with much larger quantities of free B2M. (It should be stressed that the amount of B2M administered to primates in HLA-complexed form is 500–1,000-fold less antigen than the dose of free B2M used.) Second, when an animal previously sensitised with HLA-A2 antigen was boosted after 3 yr with free B2M, high levels of anti-B2M were immediately produced, indicative of a pre-existing immunity. Third, all nonspecific cytotoxic activity can easily be removed by a B2M affinity column. In a typical experiment, the anti-HLA activity was recovered in good yield (>90%) following such treatment and was of excellent specificity (SR > 100). These results illustrate that most of the total cytotoxic activity is attributable to specific anti-HLA antibodies, and the remainder is due to nonspecific anti-B2M antibodies. Furthermore, immunosorbents prepared from B2M-absorbed primate monospecific antisera of high titre represent powerful yet simple tools for the purification of HLA antigens from crude starting material, without recourse to prolonged chromatographic separations.

Apart from the mutual augmentation effect of HLA on B2M across a close species barrier, a further interesting feature of all the primate antisera is that they uniformly fail to precipitate B2M either directly or in gel diffusion plates. This is in contrast to the rat, guinea pig, chicken and rabbit antisera, which all precipitate B2M from solution. Preliminary results based on gel filtration of antigen-antibody complexes between primate antisera and B2M indicate that the anti-B2M antibodies are directed against only two sites on the B2M molecule, regardless of whether they are produced in response to free or HLA-linked B2M. This would preclude any lattice formation necessary to produce precipitation with antigen, whereas the radioimmunoassay used here<sup>10</sup> for quantitation of anti-B2M antibodies is not dependent on direct precipitating properties of the antiserum. Rabbit antisera seem to contain antibodies



Table 2 Sensitisation of *Macaca irus* primates to human B2M

| Group | Injected protein per dose ( $\mu$ g) | B2M form*                       | Anti-B2M titre†     | Human lymphocytotoxins 50% Cytotoxic titre‡ | SR            |
|-------|--------------------------------------|---------------------------------|---------------------|---------------------------------------------|---------------|
| 1     | 50–100                               | Free                            | 93 $\pm$ 13§        | 8 $\pm$ 2                                   | 1.0           |
| 2     | 0.5–1.0¶                             | Papain-solubilised HLA antigens | 2,406 $\pm$ 1,295** | 3,250 $\pm$ 1,673                           | 4.8 $\pm$ 1.1 |

\*All injections were in incomplete Freund's adjuvant.

†See Table 1, footnote ‡.

‡See Table 1, footnote §.

§Detectable responses were only produced after six booster injections.

¶See Table 1, footnote \*\*, the same preparations were used.

||See Table 1, footnote ††.

\*\*Responses were detectable after two boosts, titres shown are after three boosts.

directed against three or four sites, and chicken sera detect at least six sites on B2M which would accord with their precipitating properties. The similarity between primate antisera raised either to free B2M or to the HLA-linked B2M in terms of precipitating properties and the number of epitopes recognised, renders it unlikely that conformational changes in B2M are imposed as a consequence of the HLA association and that this results in the augmented immunogenicity noted here.

It may be concluded from these experiments that although free B2M is quite immunogenic across wide species barriers, the immunogenicity declines when the barrier is phylogenetically close. The situation is quite different when B2M bound to HLA antigens is considered. B2M is able to derive immunogenic 'help' by associative recognition, because of its peculiar association with these MHC antigens. Although B2M is not covalently linked to HLA, the bonding is nevertheless strong, in that low pH or very strongly ionic detergents are required to separate the protein chains. The help provided by the alloantigen across a species barrier may thus be intermediate between the two types defined<sup>11,12</sup>. 'Intramolecular help' refers to a situation where helper and principal determinant are on the same molecule. "Inter-molecular, intrastructural" which may be somewhat less effective (N. A. Mitchison, personal communication), refers to helper and principal determinants being on different molecules. Because B2M and its attendant alloantigen mobilise jointly on cell surfaces ('capping'), however, and remain tightly bound when solubilised from membrane by enzyme, non-ionic, or weakly ionic detergents, they may for most purposes be considered as being on the same molecule and therefore presented as an immunogenic unit in circumstances such as tissue grafting or the experiments described here with HLA antigen.

Recent evidence (in rats<sup>13</sup>) suggests that within a species, both Ia and AgB antigens must be presented on the same living cell in order to provoke anti-AgB antibodies. Soluble alloantigens, or cellular membranes which do not express Ia antigens, are essentially non-immunogenic in a primary response. Primed animals may be different, however, and it is worth noting that once sensitisation has occurred, help can be provided for particular H2 components by other MHC gene products and/or minor non-MHC antigens<sup>14</sup>.

Immunisations across species barriers with purified HLA antigens present quite a distinct contrast. Provided there is some difference at B2M between the species, then even when donor and recipient are phylogenetically close and their MHC antigens might therefore be expected to be quite similar, the high degree of MHC polymorphism is almost certain to ensure a second (HLA) epitopic difference on the two-chain antigen molecule, thereby providing an opportunity for associative recognition and help. It should also be borne in mind that a large proportion of T lymphocytes

is able to recognise MHC antigens within a species<sup>15,16</sup>, and therefore probably to recognise analogous MHC antigens across a close phylogenetic barrier. It would be expected then that the help provided would be very substantial because of this large existing clone from which T helpers could be derived. This is proposed as the explanation for the high titres of anti-B2M antibodies found here in primates, and it occurs essentially because of the B2M-HLA association. Furthermore, the high degree of allospecificity, especially when anti-B2M antibodies are removed, suggests that the non-alloantigenic portion of the glycoprotein chain is only poorly immunogenic between related species. This conclusion was reached earlier in other primate experiments<sup>17</sup>. In dosage terms, the very small amount of soluble alloantigen is as effective outside the species as the same quantity of alloantigen presented in cellular form on viable lymphoid tissue within the species of origin. These inter-relationships are summarised diagrammatically in Fig. 1, where allogeneic, and close or distant xenogeneic immunogenicity is depicted.

In the xenogeneic (mouse-man) primed lymphocyte typing assay it has been found<sup>18</sup> that both the stimulating and target antigens are probably identical to those involved in the allogeneic mouse mixed lymphocyte reaction. The role of B2M was not examined, but when taken in conjunction with the findings reported here, it suggests that the part played by this molecule in xenograft rejection and therefore in species discrimination and interfertility will warrant further investigation.

It is also possible to imply that HLA antigens or their equivalent in other species may serve as 'super carriers' for artificially attached haptens and epitopes when the MHC barrier is crossed within a species or in a closely related species, and this implication is presently under investigation. The examination of what may thus be considered as modified 'non-self' has obvious relevance to the present interest in altered 'self' as a possible mechanism whereby animals combat viruses<sup>19</sup>.

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## In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells

CELL membrane structures controlled by genes in the major histocompatibility complex (H-2 in mice) are involved in most immune interactions between T lymphocytes and other cells<sup>1</sup>. Cytotoxic T lymphocytes (CTL) immunised against viruses<sup>2</sup>, haptens<sup>3</sup>, minor histocompatibility antigens<sup>4</sup> or tumour antigens<sup>5</sup>, are specific for self H-2 antigens as well as for the foreign antigen. But CTL are not restricted to recognising antigens in combination with only self H-2. H-2<sup>d</sup> homozygous CTL which have matured in an irradiated H-2<sup>d</sup>/H-2<sup>k</sup> host can respond to antigen plus H-2<sup>k</sup> in addition to antigen plus H-2<sup>d</sup> (refs 6–8). It is not known whether the H-2 environment in which T cells mature influences their range of specificity, that is, whether CTL from a normal mouse can respond quantitatively as well to antigen plus foreign H-2 as they do to antigen plus self H-2. These experiments were designed to test this influence. The results suggest that host H-2 antigens do exert an effect on the specificity of T-cell responses.

A single suspension of bone marrow cells from F<sub>1</sub>(BALB/c × BALB.B) (F<sub>1</sub>(C × C.B), H-2<sup>d</sup>/H-2<sup>b</sup>) mice was used to reconstitute groups of lethally irradiated parental mice, C(H-2<sup>b</sup>) ([F<sub>1</sub> → C] chimaeras) and C.B(H-2<sup>b</sup>) ([F<sub>1</sub> → C.B] chimaeras). Eight weeks later these chimaeric mice and normal F<sub>1</sub>(C × C.B) mice were primed against minor H antigens by injecting 8 × 10<sup>6</sup> F<sub>1</sub>(B10 × B10.D2) (H-2<sup>b</sup>/H-2<sup>d</sup>) spleen cells. The B10 background offers more than 30 minor histocompatibility antigenic differences that can be recognised by BALB mice<sup>9,10</sup>. Some weeks later the primed spleen cells were boosted in culture with irradiated F<sub>1</sub>(B10 × B10.D2) stimulator cells and assayed for cytotoxicity 5 d later.

Following this immunisation procedure, cells from normal F<sub>1</sub>(C × C.B) mice lysed B10 targets and B10.D2 targets almost equally (Table 1). (The two activities are mediated by separate

pools of CTL<sup>2–4</sup>) The chimaeras responded differently. In the same conditions of immunisation with F<sub>1</sub>(B10 × B10.D2) cells, they responded preferentially to the minor antigens in association with the H 2 antigens of the host. CTL from the [F<sub>1</sub> → C] chimaeras killed B10.D2 targets better than B10 targets, whereas CTL from [F<sub>1</sub> → C.B] chimaeras lysed B10 targets better than B10.D2 targets (Table 1).

Spleen cells from five chimaeras were assayed for their content of host and donor cells at time of killing. Complement-mediated lysis with H-2<sup>b</sup> anti-H-2<sup>d</sup> serum and with H-2<sup>d</sup> anti-H-2<sup>b</sup> serum indicated that in all cases at least 85% of the cells were of F<sub>1</sub> (donor) origin. The cytotoxic effector cells were also lysed with anti-H-2 serum and complement just before the <sup>51</sup>Cr-release assay (Table 2). Here, the [F<sub>1</sub> → C] chimaera cells lysed B10.D2 targets ninefold more efficiently than they lysed B10 targets (data not shown). The killer cells were treated with antiserum plus complement, washed, and assayed for lysis of labelled B10.D2. Table 2 shows, most importantly, that BALB/c anti-C57BL/6 (anti-H-2<sup>b</sup>) serum reduced the cytotoxic activity 86% compared with controls. This antiserum does not lyse BALB/c effector cells. Therefore, at least 86% of the CTL were of F<sub>1</sub> bone marrow origin.

These experiments show that H-2<sup>d</sup>/H-2<sup>b</sup> cytotoxic cells which mature in an irradiated H-2<sup>d</sup> host respond preferentially to antigens plus H-2<sup>d</sup>, whereas H-2<sup>d</sup>/H-2<sup>b</sup> cells which mature in an irradiated H-2<sup>b</sup> host respond preferentially to the same antigens in conjunction with H-2<sup>b</sup> gene products. The experiments were designed to test the 1971 Jerne hypothesis<sup>14</sup>, or a modified version of it<sup>4,15</sup>. The hypothesis accepts that a somatic theory of generation of receptor diversity is correct and proposes that self-H-2 antigens drive the diversity. Immature T cells first express an anti-self-H-2 receptor, leading to proliferation and to accumulation of V gene mutations until there is no significant reaction with self-H-2. According to this hypothesis, the receptor repertoire of A strain T cells which had matured in an A environment would be quite different from that of A strain T cells which had matured in a B environment. The results presented here are compatible with this hypothesis and with another theory of 'adaptive differentiation'<sup>1</sup>.

There is an alternative explanation of the results. It may be that the host haplotype preference seen at the level of effector CTL does not reflect a bias in specificity at the level of precursor CTL. The H-2<sup>d</sup>/H-2<sup>b</sup> precursor CTL in the H-2<sup>d</sup> host may have exactly the same range of reactivity as those in the H-2<sup>b</sup> host. The haplotype preference of the effector CTL would then be due to the way antigen is presented to CTL precursors. Even though the immunogen (B10 minor antigens) was introduced on H-2 heterozygous cells, the antigen which was responsible for priming CTL may have been processed antigen presented on radiation resistant host cells<sup>4</sup>. In the [F<sub>1</sub> → C] chimaera such radiation-resistant antigen-present-

Table 1 Specificity of H-2<sup>d</sup>/H-2<sup>b</sup> cytotoxic cells from normal and chimaeric mice

| Responder*                                                           | Immunised with†                                                    | B10<br>H-2 <sup>b</sup> | % Specific lysis of<br>B10.D2<br>H-2 <sup>d</sup> | % Specific lysis of<br>B10.BR<br>H-2 <sup>k</sup> | F <sub>1</sub> (C × C.B)<br>H-2 <sup>d</sup> /H-2 <sup>b</sup> | Ratio of lytic<br>activity on§<br>B10/B10.D2 |
|----------------------------------------------------------------------|--------------------------------------------------------------------|-------------------------|---------------------------------------------------|---------------------------------------------------|----------------------------------------------------------------|----------------------------------------------|
| Experiment 1                                                         |                                                                    |                         |                                                   |                                                   |                                                                |                                              |
| Normal F <sub>1</sub> (C × C.B)(H-2 <sup>d</sup> /H-2 <sup>b</sup> ) | F <sub>1</sub> (B10 × B10.D2)(H-2 <sup>b</sup> /H-2 <sup>d</sup> ) | 56.2                    | 62.2                                              | 1.5                                               | ND                                                             | 0.7                                          |
| Chimaera [F <sub>1</sub> → C]                                        | F <sub>1</sub> (B10 × B10.D2)(H-2 <sup>b</sup> /H-2 <sup>d</sup> ) | 19.3                    | 72.7                                              | 2.9                                               | ND                                                             | 0.02                                         |
| Chimaera [F <sub>1</sub> → C.B]                                      | F <sub>1</sub> (B10 × B10.D2)(H-2 <sup>b</sup> /H-2 <sup>d</sup> ) | 51.8                    | 29.1                                              | 0.1                                               | ND                                                             | 5.2                                          |
| Normal F <sub>1</sub> (C × C.B)                                      | C3H(H-2 <sup>k</sup> )                                             | 4.5                     | 3.8                                               | 71.5                                              | ND                                                             | —                                            |
| Experiment 2                                                         |                                                                    |                         |                                                   |                                                   |                                                                |                                              |
| Normal F <sub>1</sub> (C × C.B)                                      | F <sub>1</sub> (B10 × B10.D2)                                      | 54.8                    | 68.4                                              | ND                                                | 0.9                                                            | 0.5                                          |
| Chimaera [F <sub>1</sub> → C]                                        | F <sub>1</sub> (B10 × B10.D2)                                      | 4.8                     | 80.5                                              | ND                                                | 1.5                                                            | <0.02                                        |
| Chimaera [F <sub>1</sub> → C.B]                                      | F <sub>1</sub> (B10 × B10.D2)                                      | 61.3                    | 8.8                                               | ND                                                | 0.1                                                            | >43.0                                        |

\*Chimaeras were prepared as follows: BALB/c(C(H-2<sup>d</sup>)) and BALB.B(C.B(H-2<sup>b</sup>)) mice were irradiated with 850 R and reconstituted on the same day with 1.34 × 10<sup>6</sup> anti-Thy-1 plus complement (C')-treated F<sub>1</sub>(C × C.B) bone marrow cells. Eight weeks later they were immunised.

†Primed against minor H antigens by injecting 8 × 10<sup>6</sup> viable F<sub>1</sub>(B10 × B10.D2) spleen cells intraperitoneally. Spleen cell suspensions were prepared 4 weeks later (experiment 1) or 6 weeks later (experiment 2), and boosted for 5 d in culture with an equal number of 1,000 R irradiated F<sub>1</sub>(B10 × B10.D2) or C3HeB/F<sub>1</sub> spleen cells as described previously<sup>11</sup>.

‡2-d con A (conavalin A) blasts were labelled with <sup>51</sup>Cr-sodium chromate and used as targets as described previously<sup>11</sup>. Serial dilutions of the killers were assayed against a constant number of targets, and the figures for specific lysis presented here are for a killer: target ratio of 100:1. Spontaneous release of <sup>51</sup>Cr varied from 18.1–22% in experiment 1 and from 9.6–13.7% in experiment 2.

§Calculated from the titrations of killers: targets<sup>12,13</sup>. For example, in experiment 1, the [F<sub>1</sub> → C] cells lysed B10.D2 targets 50 times better than they lysed B10 since a 100:1 ratio caused 19.3% specific release from <sup>51</sup>Cr-B10, whereas the same amount of specific lysis of <sup>51</sup>Cr-B10.D2 was obtained at a ratio of 2:1.

ND. Not determined.

**Table 2** Sensitivity of chimaera cytotoxic cells to anti-H-2 serum plus C'

| Cytotoxic cells*                                        | Treatment of effector cells† | % Specific lysis of <sup>51</sup> Cr-B10.D2‡ | % Reduction in lytic activity§ |
|---------------------------------------------------------|------------------------------|----------------------------------------------|--------------------------------|
| [F <sub>1</sub> → C] anti-F <sub>1</sub> (B10 × B10.D2) | Medium                       | 34.0                                         | —                              |
|                                                         | Normal B10 serum + C'        | 34.3                                         | 0                              |
|                                                         | Anti-H-2 <sup>b</sup> + C'   | 9.8                                          | 86                             |
|                                                         | Anti-H-2 <sup>d</sup> + C'   | 5.1                                          | 94                             |

\*[F<sub>1</sub> → C] chimaeras were primed *in vivo* 8 weeks after reconstitution, their spleen cells boosted in culture 20 weeks later and assayed for cytotoxicity on day 5 of culture.

†BALB/c anti-C57BL/6 and C57BL/6 anti-BALB/c sera were prepared by hyperimmunisation with spleen cells. Effector cells were incubated with mouse sera 1:2, washed, and incubated in guinea pig serum 1:9 as a source of complement (C'). Cells were re-suspended to the same volume and assayed.

‡Con A blasts from B10.D2 mice were labelled with <sup>51</sup>Cr and used as target. Data presented are for original number of responder spleen cells:target cells of 7:1. Spontaneous release of <sup>51</sup>Cr was 20.6%.

§Calculated from the titrations of killer:targets as in Table 1.

ing cells would be homozygous H-2<sup>d</sup> and would naturally stimulate only anti-B10.D2(H-2<sup>d</sup>) CTL, not anti-B10(H-2<sup>b</sup>) CTL. Experiments to decide between these interpretations are in progress.

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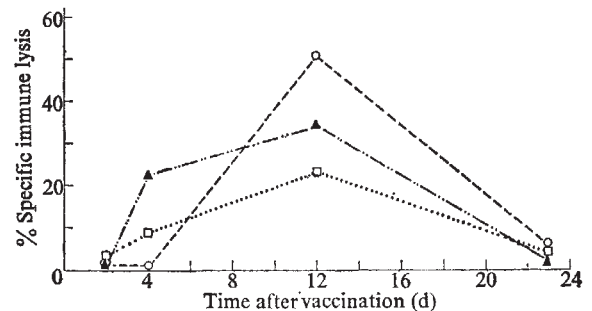
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## Cytotoxic T lymphocytes induced in mice by inactivated influenza virus vaccine

THERE is abundant evidence that T cell-mediated lysis of virus-infected target cells generally requires H-2K or H-2D region histocompatibility between cytotoxic T lymphocytes (CTL) and target cells<sup>1–6</sup>. The biological basis of this requirement is still uncertain. Studies using chimaeric mice suggest that T lymphocytes are selectively sensitised by the viral and H-2 antigens expressed on the infected cell and that killing only occurs when the target cell shares H-2K or H-2D identity with the immunising virus-infected cell<sup>7</sup>. It is important to determine whether virus infection of the host tissue is, in fact, necessary for specific CTL immunity to develop. If this were so, the use of inactivated virus or purified viral antigens would not be suitable as vaccines against diseases in which CTL were required to afford optimal protection. We report here that inactivated influenza virus vaccines are, in fact, quite capable of evoking haemagglutinin specific CTL.

Epidemiological and experimental evidence indicate that protective immunity against influenza virus is principally directed against the virus haemagglutinin<sup>8,9</sup>. We have recently described the development of haemagglutinin-specific CTL in influenza-infected mice<sup>10</sup>. This finding is consistent with these cells playing an important *in vivo* function in influenza immunity. Our finding contrasts with



**Fig. 1** Specific immune lysis by spleen cells of BALB/c mice immunised intraperitoneally with 5,000 chick cell haemagglutinating units (HAU), (○), 500 HAU (▲) and 50 HAU (□) of A/Port Chalmers formalin-inactivated whole virus influenza vaccine. The spleen cells were tested at a 100:1 ratio on A/Port Chalmers-infected syngeneic kidney-derived cells in an 18-h <sup>51</sup>Cr-release cytotoxicity assay as described previously<sup>10</sup>. The cytotoxic cells generated in vaccine-immunised mice were shown to be T cells by the removal of cytotoxic activity using anti-θ antiserum plus complement. Thus, whereas untreated spleen cells and spleen cells treated with complement alone achieved 42.3 and 37.0% specific lysis respectively, spleen cells treated with anti-θ antiserum plus complement achieved 7.9% specific lysis, which was not significant ( $P > 0.5$ ).

recent studies which failed to demonstrate haemagglutinin-specific CTL in influenza-infected mice<sup>11–13</sup>. These studies differed from our study, however, in the use as target cells of relatively non-permissive influenza-infected transformed cell lines rather than productively-infected normal tissue-derived target cells<sup>14</sup>. In the present study we have similarly tested mice inoculated with influenza vaccine, for haemagglutinin-specific CTL reactive with productively-infected target cells. The virus vaccines used contained an early H3N2 virus strain<sup>9</sup>, A/Aichi/68; a later virus of the same H3N2 subtype, but with a serologically distinguishable haemagglutinin, A/Port Chalmers/73; and an unrelated influenza B virus, B/Hong Kong/72. The target cells were syngeneic kidney-derived infected cells<sup>10</sup>. The experiment was carried out in both BALB/c mice (H-2<sup>d</sup>) and C3H mice (H-2<sup>k</sup>).

Figure 1 depicts the CTL response we observed in BALB/c mice inoculated intraperitoneally with various doses of A/Port Chalmers vaccine (virus vaccine supplied by Merrell-National Laboratories). Significant cytotoxic T-cell response was observed with each of the doses tested. To determine whether the response was restricted to target cells histocompatible with the immunised mice, we tested spleen cells from BALB/c and C3H immunised mice on syngeneic and allogeneic virus-infected target cells. As indicated in Table 1, the cytotoxic response was detectable only on the syngeneic virus-infected target cells. Specificity of the CTL for the haemagglutinin antigen of the immunising vaccine virus was shown in the experiments recorded in Table 2. Thus, CTL-distinguished target cells infected with the immunising strain H3N2 influenza virus, from target cells infected with a serologically different but related

**Table 1** Virus specificity of cell-mediated lysis by CTL in influenza vaccine immunised mice

| Vaccine virus used as immunogen | Specific immune lysis of target cells* |                  |                      |
|---------------------------------|----------------------------------------|------------------|----------------------|
|                                 | A/Port Chalmers-infected               | A/Aichi-infected | B/Hong Kong-infected |
| A/Port Chalmers/73              | 96.6                                   | 6.2              | 8.8                  |
| A/Aichi/68                      | 5.2                                    | 67.0             | 2.7                  |
| B/Hong Kong/72                  | —6.9                                   | 3.2              | 63.4                 |

\*Spleenic lymphocytes were tested 8 d after intraperitoneal immunisation.



**Table 2** Allogeneic restriction of cell-mediated lysis by CTL in mice immunised with influenza vaccine

| Source of immune lymphocytes* | C3H target cells | BALB/c target cells |
|-------------------------------|------------------|---------------------|
| BALB/c                        | 1.7              | 21.3                |
| C3H                           | 33.4             | 1.9                 |

\*Splenic lymphocytes were tested 5 d after intraperitoneal immunisation.

H3N2 strain. Increasing knowledge is becoming available concerning the structure of the influenza virus haemagglutinin antigen. It should be possible, therefore, to extend the studies reported in this paper to define the precise cellular-antigen interactions involved in the generation of CTL and to determine the basis of the H-2 compatibility requirement between CTL and virus-infected target cells.

The finding that inactivated influenza virus vaccine can induce a haemagglutinin-specific CTL response is in agreement with the recent finding of Schrader and Edelman<sup>15</sup> that inactivated Sendai virus can elicit CTL in primary cultures of mouse spleen cells<sup>11</sup>, and the observation of Wiktor *et al.* that CTL develop in mice inoculated with inactivated rabies virus<sup>16</sup>. Together, these findings provide considerable support to the potential development of purified protein vaccines for use against chronic viral diseases and malignancy where T cell-mediated, rather than humoral, immunity seems to be deficient.

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## Induction of plaque-forming cells in cultured human lymphocytes by combined action of antigen and EB virus

PRIMARY *in vitro* immune responses of human peripheral blood lymphocytes (PBL), desirable for both basic and clinical studies, have been rather difficult to achieve. Positive results were obtained when other stimulatory factors, besides antigen, were given to the cells<sup>1,2</sup>, and other reports have suggested a critical role of antigen presentation and antigen concentration<sup>3,4</sup>. In view of the difficulty encountered in stimulating an antibody response with antigen alone,

we have attempted to use Epstein-Barr virus (EBV) together with sheep red blood cells (SRBC) to induce a specific response. Human B lymphocytes possess membrane receptors for EBV<sup>5,6</sup> and the virus stimulates DNA synthesis in human PBL<sup>7</sup>, so EBV provides a 'nonspecific' stimulus in addition to the specific one given by the antigen. We report here that use of EBV and SRBC in this way consistently induces an anti-SRBC response from human PBL.

Human PBL, from different donors, were cultured in the conditions previously described<sup>2</sup>. The source of the virus was the EBV-secreting marmoset lymphoid cell line B 95-8 (ref. 8). Table 1 shows that the appearance of plaque-forming cells (PFC) was a rare and inconsistent event when human PBL were cultured in the presence of SRBC only. Addition of EBV alone led, with some donors, to a measurable but transient appearance of background PFC. But, when both antigen and virus were present in the culture from the very beginning a significant immune response was regularly observed with the majority of donors. Ultraviolet inactivation of the virus did not abrogate this effect. There was no stimulation if the addition of the virus was delayed by 48 h, or if culture supernatant freed of virus was added.

To determine whether the PFC responses were antigen specific, either SRBC or horse red blood cells (HRBC) were added to cultures in the presence of EBV. Each culture was screened separately for PFC using SRBC and HRBC as indicator cells. The results in Table 2 show that the responses obtained were specific for the antigen added. The occasional appearance of nonspecific PFC with some donors could be due either to an antigen-independent response induced by EBV or to a cross-reactivity between the two antigens.

Though the basic phenomenon described above is remarkably reproducible, the kinetics and height of the response vary considerably. PFC levels were very low before day 4 and usually reached a maximum between days 7 and 9. While in some cases PFC were present over 2 or 3 days only, cells of other donors yielded PFC in high numbers over 1 week or more. In some experiments a second burst of PFC (occasionally amounting up to a small percentage of

**Table 1** Dependence of anti-SRBC response on antigen and virus

| Donor | EBV | SRBC | PFC per well |       |
|-------|-----|------|--------------|-------|
|       |     |      | Day 7        | Day 9 |
| LG    | +   | +    | 292          | 106   |
|       | —   | +    | 4            | 0     |
|       | +   | —    | 90           | 0     |
| JH    | +   | +    | 361          | 167   |
|       | —   | +    | 1            | 3     |
|       | +   | —    | 39           | 6     |
| AW    | +   | +    | 250          | 1953  |
|       | —   | +    | 0            | 0     |
|       | +   | —    | 0            | 0     |
| JB    | +   | +    | 853          | 973   |
|       | —   | +    | 0            | 4     |
|       | +   | —    | 426          | 60    |

Human lymphocytes were prepared from heparinised blood by centrifugation on a Ficoll-Urovison gradient. Adherent cells were removed and the non-adherent cells were suspended at a density of  $9 \times 10^5$  per ml in tissue culture medium according to previously described methods<sup>2</sup> with a few modifications. Foetal calf serum was substituted with human AB serum, heat inactivated and absorbed three times in the cold with SRBC. The concentration of AB serum was 8% in the medium and 10% in the nutritional mixture. Antigen was added (0.05 ml of 1% SRBC per ml of culture) and the cell suspension was distributed in aliquots of 0.1 ml in the wells of Micro-test plates: 50  $\mu$ l of 10-fold concentrated virus-stock<sup>8</sup> were added to each well. The cultures were incubated at 37 °C in the presence of 5% CO<sub>2</sub>, and 24 h after initiation 50  $\mu$ l of nutritional mixture were placed in each well. At the time of assay the content of five wells was pooled, washed and plaqued with a modification of the haemolysis in gel method<sup>2</sup>. Figures represent PFC per well.

Table 2 Antigen specificity of the EBV-assisted response

| Donor | Antigen in culture | Day 7 |      | Day 8 |      | Day 10 |       |
|-------|--------------------|-------|------|-------|------|--------|-------|
|       |                    | SRBC  | HRBC | SRBC  | HRBC | SRBC   | HRBC  |
| BM    | SRBC               | 238   | 9    | 580   | 334  | 13     | 2     |
|       | HRBC               | 33    | 298  | 25    | 858  | 28     | 244   |
| JR    | SRBC               | 1,125 | 6    | 1,410 | 0    | 574    | 0     |
|       | HRBC               | 4     | 117  | 18    | 50   | 7      | 5     |
| LG    | SRBC               | 1,422 | 0    | 910   | 0    | 2,200  | 0     |
|       | HRBC               | 122   | 318  | 71    | 166  | 0      | 89    |
| CP    | SRBC               | 1,134 | 7    | 6,400 | 18   | 7,500  | 2     |
|       | HRBC               | 250   | 741  | 79    | 167  | 33     | 8     |
| PC    | SRBC               | 59    | 0    | 41    | 0    | 122    | 0     |
|       | HRBC               | 5     | 12   | 0     | 55   | 0      | 2,400 |

Procedure as in the legend to Table 1. The cultures stimulated with HRBC received 0.05 ml of 1% HRBC per ml of culture. The AB serum used for these cultures was absorbed three times in the cold with HRBC. Figures represent PFC per well.

the recovered viable cells) arose beyond day 10, suggesting a biphasic event (data not shown). Moreover, comparing different culture wells of the same preparation, large variability was observed in some experiments but not in others. There were also significant differences between donors: among the 35 tested, some gave consistently high responses in up to six experiments over a period of 8 months, while others responded poorly and a few not at all. Whether this is due to technical reasons or reflects differences in the immune status of the donor is under investigation. There is apparently no correlation between the capacity to respond in the present system and previous exposure to EBV, as judged by the presence of anti-viral antibodies in the serum.

The mechanism of action of EBV is a matter for speculation, but any model would have to consider either direct action of the virus on B cells or induction of helper T cells.

In the presence of antigen the binding of EBV to its receptor could provide a sufficient signal for B-cell triggering, and bypass the requirement for T-cell help. In its extreme form this concept would attribute to the EBV receptor a role in the binding of physiological T-cell factor(s)<sup>8</sup>. But, what we observed could just as well be an instance of a much more general phenomenon: the threshold for B-cell induction is lowered by the binding of some agent to its appropriate receptor on the cell surface. It has been shown<sup>10</sup> that binding of an antibody directed against a surface component on mature murine B lymphocytes markedly enhances the appearance of PFC in conditions of suboptimal antigen doses and can substitute for T-helper activity. Furthermore, EBV can act as a polyclonal activator<sup>11</sup>. Its mode of action may therefore be analogous to the stimulatory effect of lipopolysaccharide (LPS) on the immune response to various antigens in mice<sup>12</sup>.

The alternative model implies that EBV indirectly activates T cells which would then provide, in the presence of antigen, sufficient help for B-cell induction. Support for this interpretation comes from the clinical syndrome of infectious mononucleosis. During the acute phase of this EBV-induced disease mostly thymus-derived lymphocytes proliferate and appear in the peripheral blood and in the T-dependent areas of lymphnodes<sup>13</sup>. It seems that the stimulus for the T-cell response comes from virus-determined structures on B cells<sup>9</sup>. A similar mechanism in our *in vitro* system could provide sufficient T-cell help to promote the specific antigen-dependent response. Experiments are in progress to determine the mode of action of EBV by culturing separately purified populations of T and B lymphocytes.

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## EB virus-induced B lymphocyte cell lines producing specific antibody

EPSTEIN-BARR virus (EBV) is a lymphotropic herpesvirus that converts normal human B lymphocytes into established lines. This 'immortalisation' preserves the characteristics of the original B cell, including EBV receptors, complement receptors, surface immunoglobulin and secretory immunoglobulin. Surface immunoglobulin is most frequently IgM. EBV-immortalised lines carry multiple copies of the viral genome, detected by nucleic acid hybridisation, and regularly express an EBV-specific nuclear antigen, EBNA. It has been suggested<sup>1</sup> that all B-cells carry EBV receptors. If so, and if all B cells can be transformed into lines by EBV, it should be feasible to establish permanent lines from B lymphocytes capable of producing specific antibodies against appropriate antigens. Clearly, in view of the polyclonal transformation obtained after infection of B lymphocytes with a transforming virus strain, for example, B95-8, it will be necessary to pre-select lymphocytes with the appropriate antigen combining site. Rosetting with antigen-coupled erythrocytes is one of the conceivable methods for such pre-selection. While this will select lymphocytes with the appropriate surface immunoglobulin receptors, it was recently shown that EBV-transformation activates normal lymphocytes and induces the release of secretory immunoglobulin<sup>2</sup>. If the procedure is successful, the established lines might therefore be expected to carry the appropriate surface receptor and also to secrete the corresponding antibody. This was actually found in this study, opening the way to the establishment of cell lines producing specific antibodies of choice.

We have selected as lymphocyte donors three members of the laboratory staff in Helsinki who were shown to have relatively high natural antibody titres against the synthetic hapten NNP (4-hydroxy-3,5-dinitrophenacetic acid) and whose peripheral blood lymphocytes showed specific NNP rosetting.

Lymphocytes were isolated on Ficoll<sup>3</sup> and rosetted with autologous-NNP coupled erythrocytes<sup>4</sup>. NNP-azide, prepared according to Brownstone *et al.*<sup>5</sup> was coupled to erythrocytes using a hapten concentration of 2 mg ml<sup>-1</sup> in the final conjugation mixture with the erythrocytes<sup>6</sup>. The percentage of rosettes ranged between 0.08 and 0.6% of the total lymphocytes. The rosettes were spun down on Ficoll-Isopaque (density 1.077) for 20 min at 400g to remove the non-rosetting cells, and then infected with B95-8-derived EBV. As the virus source, we used supernatants of

**Table 1** Anti-NNP rosette and plaque formation by EBV-transformed lines derived from NNP rosetting and unfractionated cells, respectively

| Cell lines     | Source of EBV-transformed explant | % RFC* | % PFC† | % Ig positive cells‡ | EBNA staining§ |
|----------------|-----------------------------------|--------|--------|----------------------|----------------|
| EP             | NNP selected                      | 15     | 11     | 100                  | +              |
| LP             | NNP selected                      | 13     | ND     | 100                  | +              |
| SP             | NNP selected                      | 18     | ND     | 100                  | +              |
| S (Unf)        | Unfractionated                    | 0      | 0      | 100                  | +              |
| L (Sup)        | NNP rosette deprived              | 0      | 0      | 100                  | +              |
| E (Unf)        | Unfractionated                    | 0      | 0      | 100                  | +              |
| EP re-selected | NNP re-selected                   | 86     | 69     | 100                  | +              |

\*Rosette-forming cells (RFC):  $5 \times 10^6$  cells in 1 ml were washed and mixed with 0.01 ml human NNP-coupled erythrocytes, spun down for 15 min by low velocity centrifugation at 4 °C, and incubated further for 30 min at 4 °C. The % of rosettes was assessed after resuspension of the pelleted cells. No rosettes were formed with uncoupled erythrocytes.

†Plaque-forming cells (PFC): equal volumes of 1/5 guinea pig serum, 10% NNP-coupled erythrocytes and different numbers of cells were mixed and inserted between two slides<sup>8</sup>. Plaques were counted 90 min later. The plaques were inhibited if  $10^{-4}$  M  $\text{NaN}_3$  was added to the mixture. No plaques were detected with uncoupled erythrocytes. ND, not determined.

‡Cells were washed and stained with FITC-labelled rabbit anti-human Ig.

§EBV-determined nuclear antigen, stained according to Reedman and Klein<sup>13</sup>. Staining was 90–100% in all positive cultures.

||EP, LP, and SP are NNP-receptor positive lines established from three donors (E, L, and S) as described in the text. S(Unf) and E(Unf) are lines established from EBV infected unfractionated lymphocytes of donor S and E. L (Sup) is a line established from EBV infected lymphocytes, deprived of NNP-receptor positive cells. 'EP re-selected' is a line re-selected by NNP-erythrocyte rosetting of the EP line.

mycoplasma-free B95-8 cultures: 1 ml of the supernatant induced 20% EBNA positive cells in  $10^6$  cells of the BJAB line<sup>7</sup> after 48 h incubation. As controls we infected either unfractionated lymphocytes from the same donor or the NNP-rosette-deprived fraction. The infected cells were incubated in RPMI-20% foetal calf serum, at 37 °C, 5%  $\text{CO}_2$ . Permanent lines were established from all three donors after approximately 4 weeks. All viable cells of all lines were brilliantly EBNA positive and grew with a doubling time of approximately 24 h.

The established lines were tested for rosette formation and for plaque formation with NNP-coupled human erythrocytes<sup>8</sup>. The supernatants were assayed for specific NNP agglutination titre and for inhibition of plaque formation with 4-hydroxy-5-iodo-3-nitrophenacetic acid coupled T4 phage (NIP-cap-T4) (refs 9, 10). NIP show >90% cross

reactivity with the NNP-hapten<sup>11</sup>. The immunoglobulin class of the product was assayed by sucrose gradient sedimentation and by incubating NNP-coupled erythrocytes in the supernatants from the different lines, followed by staining with FITC-labelled anti-human immunoglobulin sera.

As shown in Table 1, the cell lines derived from the EBV transformation of the NNP-rosetting fraction contained 13–18% NNP rosetting cells, whereas the lines derived from unfractionated cells or cells remaining in the supernatant after NNP rosetting gave no specific rosettes. EP line was re-selected by renewed rosetting. In this re-selected line, the frequency of the rosette forming cells rose to 86%. The same line also formed 69% NNP-specific plaques. Plaque formation was inhibited by sodium azide, confirming that it must have been due to active secretion. Since the frequency of plaque-forming cells nearly equalled the

**Table 2** Summary of anti-NNP antibody tests with culture supernatants

| Supernatant*   | Source of EBV-transformed explant | NNP-Agglutination titre† | Haptenated phage inactivation titre | Haptenated phage inactivation titre after 2ME treatment§ | NNP-coupled erythrocytes exposed to culture supernatants, stained for |     |     |        |       |
|----------------|-----------------------------------|--------------------------|-------------------------------------|----------------------------------------------------------|-----------------------------------------------------------------------|-----|-----|--------|-------|
|                |                                   |                          |                                     |                                                          | Ig                                                                    | IgM | IgG | lambda | kappa |
| EP             | NNP selected                      | 256                      | ND                                  | ND                                                       | +                                                                     | +   | —   | —      | +     |
| LP             | NNP selected                      | 64                       | 86                                  | <3                                                       | +                                                                     | +   | —   | —      | +     |
| SP             | NNP selected                      | 64                       | 981                                 | <3                                                       | +                                                                     | +   | —   | —      | +     |
| E (Unf)        | Unfractionated                    | 0                        | ND                                  | ND                                                       | —                                                                     | —   | —   | —      | —     |
| L (Sup)        | NNP deprived                      | 0                        | ND                                  | ND                                                       | —                                                                     | —   | —   | —      | —     |
| S (Unf)        | Unfractionated                    | 0                        | ND                                  | ND                                                       | —                                                                     | —   | —   | —      | —     |
| EP re-selected | NNP re-selected                   | 1,024                    | 4,070                               | <3                                                       | +                                                                     | +   | —   | —      | +     |
| RPMI-10% FCS   |                                   | 0                        | 8                                   | <3                                                       | —                                                                     | —   | —   | —      | —     |

\*As in Table 1. ND, not determined.

†Aliquots of 0.1 ml of twofold dilutions of 0.22- $\mu\text{m}$  filtered supernatants in BSS were mixed with 0.02 ml of 2% NNP-coupled erythrocytes. Agglutination was read after 60 min at 37 °C. No agglutination was detected with uncoupled erythrocytes.

‡Aliquots of 0.3 ml of supernatant dilutions (filtered through 0.22- $\mu\text{m}$  filter) were mixed with 0.3 ml of NIP-cap-T<sub>4</sub> suspension containing 200 PFU (plaque-forming units) and incubated for 4 h at 37 °C. 5 ml 0.7% fluid agar containing about  $10^8$  *Escherichia coli* was then added, poured on to a solid 2% agar plate, and incubated overnight at 37 °C. The titres shown are the reciprocals of the dilution causing 50% inactivation of haptenated phages.

§Supernatants were treated with 0.3 M 2-mercaptoethanol for 60 min at 37 °C before mixing with the NIP-cap-T<sub>4</sub> phage.

||NNP-coupled erythrocytes were incubated with the culture supernatants for 60 min, at 37 °C, washed and stained with FITC conjugated antisera. Uncoupled erythrocytes showed no staining after the same treatment. The following FITC conjugates were used: (1) rabbit anti-human IgG, IgA, IgM, kappa, lambda; (2) anti-IgM, specific for  $\mu$  chains; (3) anti-IgG specific for Fc fragment; (4) anti-kappa, light chains (Bence-Jones); (5) anti-lambda, light chains (Bence-Jones). They were all products of Dakopatts, Denmark. The following cell lines were included as positive and negative controls in each test; BJAB<sup>14</sup>: IgM-kappa positive; Ramos<sup>14</sup>: IgM-lambda positive; Rael<sup>15</sup>: IgG-lambda positive.



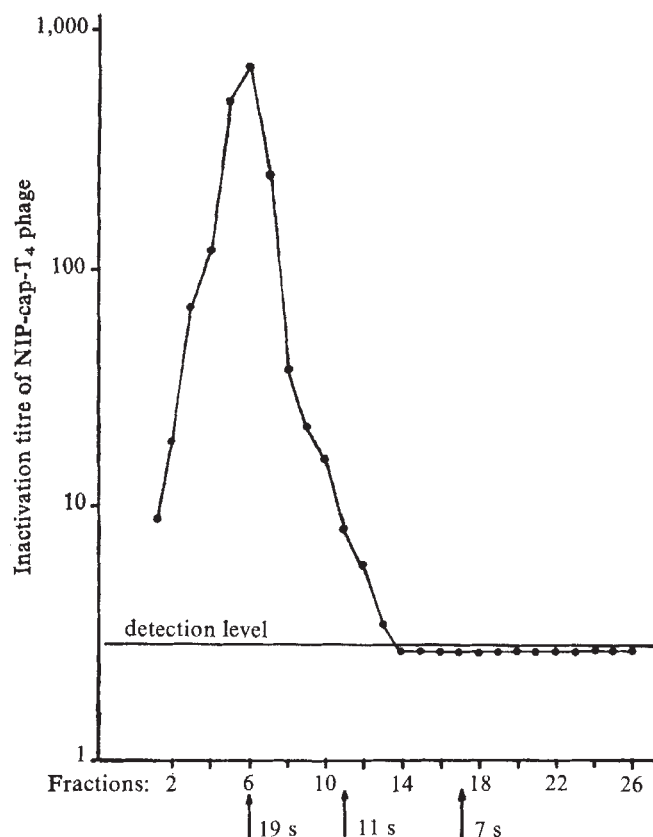


Fig. 1 EP culture supernatant was run on 5–20% sucrose gradient, Beckman SV41 39,000 r.p.m. for 13 h. The fractions were tested for inhibition of NIP-cap-T<sub>4</sub> phage plaque formation on *E. coli* as described in Table 2. NIP shows > 90% cross reactivity with the NNP hapten.

frequency of rosette-forming cells, the PFC and RFC forming populations were either identical or largely overlapping.

The agglutination titre of the supernatants ranged between 64 and 1,024. The 50% plaque inhibition titre with the NIP-cap-T<sub>4</sub> phage ranged between 86 and 4,070. All inhibitory activity was destroyed after treating the supernatant with 2-mercaptoethanol, indicating that the antibody was IgM, or at least not IgG.

Table 2 shows the results of all immunological tests, including staining of NNP-coupled erythrocytes with the culture supernatants, followed by FITC-conjugated anti-immunoglobulin reagents, as indicated. All three NNP-selected, transformed lines released anti-IgM-kappa antibody, according to the erythrocyte staining test.

Figure 1 shows a sucrose gradient pattern of the EP supernatant. It is clear that the specific plaque inhibition peaked as 19S. This is in line with the NNP-coupled erythrocyte staining test.

Since EBV-transformed lymphoblastoid cell lines are polyclonal for a long time after their establishment<sup>12</sup>, the lines will have to be cloned before it is possible to say whether 100% antibody producing cells can be obtained in this way.

These results show that specific antibody-forming human cell lines can be established by a combination of B-lymphocyte selection, based on specific antigen affinity, and subsequent EBV immortalisation. It should be possible to manufacture lines, selected for the production of particularly interesting antibodies. Monospecific anti-HLA activity would be an obvious example.

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## Interferon-induced transfer of viral resistance between animal cells

MANY animal cell types exhibit the ability to communicate between themselves, both *in vivo* and *in vitro*<sup>1</sup>. This phenomenon is thought to occur through gap junctions which allow cells to share their metabolites and small control molecules<sup>2–6</sup>. The overall effect of this cellular communication seems to be a coordinated control of growth and function of different cells and cell types. Interferon is believed to act at the cell membrane in a fashion similar to polypeptide hormones<sup>7–9</sup>. This membrane interaction in turn leads to de-repression and production of the antiviral protein<sup>10,11</sup>. If, as is the case with polypeptide hormones<sup>12</sup>, the induction of the antiviral protein is mediated through secondary molecules, these might influence adjacent cells. The presence of such intermediary molecules during interferon induction of the antiviral protein has been inferred from kinetic studies<sup>13,14</sup>. The many instances of the species specificity of interferon action<sup>15</sup> make this a testable hypothesis. We show here that cells made resistant to virus infection by treatment with their homologous interferon can transfer viral resistance to cells of a heterologous species insensitive to that interferon and also to cells of the homologous species.

The experimental plan was to determine whether interferon treated mouse L cells could transfer their antiviral resistance to co-cultivated heterologous cells. Mouse L cells were cultured in various ratios with either human (WISH) or baby hamster kidney (BHK) cells in the presence or absence of mouse C243 cell interferon. Controls consisted of an equivalent total number of either cell species alone in the presence or absence of mouse interferon.

Table 1 shows that whereas WISH or BHK cells alone are not sensitive to the action of mouse interferon, co-cultivation of these cells in the presence of mouse interferon and sensitive mouse L cells resulted in a marked inhibition of the expected yield of virus from the interferon-insensitive cells. Control cell mixtures, in the absence of interferon (Table 1, experiments 1 and 3), yielded at least the expected amount of virus as compared with virus yield from either cell type alone. These data indicate

Table 1 Interferon-induced transfer of viral resistance between heterologous animal cells

| Cell ratio<br>( $2.25 \times 10^6$<br>cells per well) | Mouse interferon<br>( $7,500 \text{ PDD}_{50} \text{ U ml}^{-1}$ )* | Experiment 1                              |                                           |                                  | Experiment 2                              |                                           |                                  | Experiment 3                              |                                           |                                  |
|-------------------------------------------------------|---------------------------------------------------------------------|-------------------------------------------|-------------------------------------------|----------------------------------|-------------------------------------------|-------------------------------------------|----------------------------------|-------------------------------------------|-------------------------------------------|----------------------------------|
|                                                       |                                                                     | log <sub>10</sub> virus yield<br>Observed | log <sub>10</sub> virus yield<br>Expected | log <sub>10</sub><br>inhibition† | log <sub>10</sub> virus yield<br>Observed | log <sub>10</sub> virus yield<br>Expected | log <sub>10</sub><br>inhibition† | log <sub>10</sub> virus yield<br>Observed | log <sub>10</sub> virus yield<br>Expected | log <sub>10</sub><br>inhibition† |
| L cell: WISH (2 : 1)                                  | +                                                                   | ND                                        |                                           |                                  | 4.2                                       | 5.28                                      | 1.08                             | ND                                        |                                           |                                  |
|                                                       | —                                                                   | ND                                        |                                           |                                  | ND                                        |                                           |                                  | ND                                        |                                           |                                  |
| L cell: WISH (1 : 1)                                  | +                                                                   | 3.80                                      | 5.75                                      | 1.95                             | 4.5                                       | 5.46                                      | 0.96                             | 4.88                                      | 6.63                                      | 1.75                             |
|                                                       | —                                                                   | 6.05                                      |                                           |                                  | ND                                        |                                           |                                  | 6.93                                      |                                           |                                  |
| L cell: WISH (1 : 2)                                  | +                                                                   | ND                                        |                                           |                                  | 4.98                                      | 5.58                                      | 0.60                             | ND                                        |                                           |                                  |
|                                                       | —                                                                   | ND                                        |                                           |                                  | ND                                        |                                           |                                  | ND                                        |                                           |                                  |
| L cell: BHK (2 : 1)                                   | +                                                                   | 3.00                                      | 5.28                                      | 2.28                             | ND                                        |                                           |                                  | ND                                        |                                           |                                  |
|                                                       | —                                                                   | 5.76                                      |                                           |                                  | ND                                        |                                           |                                  | ND                                        |                                           |                                  |
| L cell: BHK (1 : 1)                                   | +                                                                   | 3.70                                      | 5.41                                      | 1.71                             | ND                                        |                                           |                                  | 4.41                                      | 6.55                                      | 2.14                             |
|                                                       | —                                                                   | 5.71                                      |                                           |                                  | ND                                        |                                           |                                  | 6.85                                      |                                           |                                  |
| L cell                                                | +                                                                   | < 3.00                                    |                                           | > 2.69                           | < 4.5                                     |                                           | > 1.47                           | < 3.00                                    |                                           | > 3.95                           |
|                                                       | —                                                                   | 5.69                                      |                                           |                                  | 5.97                                      |                                           |                                  | 6.95                                      |                                           |                                  |
| WISH                                                  | +                                                                   | 5.16                                      |                                           | 0.19                             | 5.76                                      |                                           | 0.07                             | 6.00                                      |                                           | 0.11                             |
|                                                       | —                                                                   | 5.35                                      |                                           |                                  | 5.83                                      |                                           |                                  | 6.11                                      |                                           |                                  |
| BHK                                                   | +                                                                   | 5.37                                      |                                           | -0.02                            | ND                                        |                                           |                                  | 6.47                                      |                                           | 0.08                             |
|                                                       | —                                                                   | 5.35                                      |                                           |                                  | ND                                        |                                           |                                  | 6.59                                      |                                           |                                  |

Mouse L cells and human WISH cells or baby hamster kidney (BHK) cells in Eagle's medium (supplemented with 10% foetal calf serum) were cultured in various ratios in Micro Test II tissue-culture plates (Falcon Plastics, Oxnard, California). The total number of cells in each well (about  $28 \text{ mm}^2$ ) was  $2.25 \times 10^6$ . Controls consisted of an equivalent total number of either cell species alone. Interferon (final concentration  $7,500 \text{ PDD}_{50} \text{ U ml}^{-1}$ ) or an equal volume of medium was added; cultures were incubated overnight at  $37^\circ \text{C}$  in a 4%  $\text{CO}_2$  atmosphere. Supernatant fluids were decanted and each well infected with  $5 \times 10^4$  p.f.u. of VSV. After 1.5 h at  $37^\circ \text{C}$ , inoculum was decanted, cell sheets were washed once and replenished with fresh medium. Virus yields from pooled triplicate cultures were determined approximately 24 h later by a slightly modified microplaque assay in which methylcellulose (0.5%) was substituted for carboxymethylcellulose<sup>19</sup>. Since interferon-treated L cells produce a negligible amount of virus, the expected yield is calculated from the percentage of the WISH cells in a cell mix without interferon (experiments 1 and 3) or a percentage of the heterologous cell yield with interferon (experiment 2).

\*The 50% plaque-depressing dose ( $\text{PDD}_{50}$ ) is defined as the amount of an interferon preparation that inhibited 50% of the plaques from developing as compared with controls. In our assay system, i U of National Institutes of Health (NIH) mouse reference interferon preparation had a titre of 2  $\text{PDD}_{50} \text{ U}$ .

†Any difference greater than  $0.5 \log_{10}$  is significant at  $P < 0.05$ .

that the presence of interferon with its homologous cell can induce antiviral activity in heterologous cells. The controls show that inhibition of virus yield does not result from co-cultivation of different cell species but results from the presence of the mouse interferon preparation with the co-cultivated cells. In addition, the degree of inhibition of virus yield in mixed cultures in the presence of mouse interferon seems to be directly related to the ratio of L cells to WISH or BHK cells (Table 1, experiments 1 and 2); that is, the higher the proportion of L cells the more inhibition of virus yield from the heterologous cells.

Initially, it was important to determine that the observed transfer of viral resistance from L cells to heterologous cell species was initiated by mouse interferon and also that the transferred resistance had the characteristics of interferon action.

Table 2 shows that when partially purified mouse L-cell interferon,  $100 \text{ PDD}_{50} \text{ U ml}^{-1}$  ( $10^7 \text{ U per mg protein}$ ), were incubated with a 1:1 mix of L and WISH cells there was 90% inhibition of the expected virus yield. Thus a partially purified interferon preparation caused transfer of resistance. Further, interferon produced by mouse L cells was as effective as that produced in C243 cells.

One hallmark of interferon action is its sensitivity to inhibition by actinomycin D. L cells were treated for 1 h at room temperature with actinomycin D  $5 \mu\text{g ml}^{-1}$ , washed to remove the unbound antibiotic and mixed with an equal number of untreated WISH cells in the presence or absence of mouse interferon. Control experiments had shown that actinomycin D  $2 \mu\text{g ml}^{-1}$  caused 97% inhibition of RNA synthesis in 15 min. As seen in Table 2, actinomycin D blocked both the action of mouse interferon on L cells and the ability of L cells to transfer viral resistance to WISH cells.

Another criterion of interferon action is its inhibitory effect on a wide range of viruses. At the low multiplicity of infection (MOI) usually used, vaccinia virus was inhibited by mouse interferon in L cells alone (98.5%) as

well as in the 1:1 L cell: WISH cell mixture (93.3%) (Table 2). In higher MOI conditions ( $5 \times 10^4$  p.f.u. per well), vaccinia virus was not sensitive to mouse interferon in L cells alone, and transfer of resistance to WISH cells was not observed (data not shown). In our experience, therefore, the susceptibility of vaccinia virus to mouse interferon in L cells is highly dependent on the input MOI. Likewise, the ability of L cells to transfer viral resistance to WISH cells was similarly dependent on the input MOI. These data also show that interferon induced transfer of viral resistance can be demonstrated for both a DNA (vaccinia virus) and a RNA (vesicular stomatitis virus, VSV) virus in parallel with their sensitivity to interferon.

Taken together, Table 2 results strongly suggest that initiation of the transfer process was due to mouse interferon, since it occurred with crude as well as highly purified interferon preparations. Its action was on the cell rather than the virus, since resistance was manifested in the absence of interferon but in the presence of cells which had been acted on by interferon; and the transfer process was initiated by mouse interferon produced in two different cell lines (L and C243). These two interferon preparations have been shown to have all the classic characteristics of interferon (that is, species specificity, broad virus specificity, requirement of RNA and protein synthesis for action, inhibition by anti-mouse interferon antisera, non-dialysability, trypsin sensitivity, pH 2 resistance and so on)<sup>15</sup>. The results also indicate that transferred resistance has the characteristics of the interferon system in that it was blocked by actinomycin D, was effective against both a RNA (VSV) and a DNA (vaccinia virus) virus, and that the level of transferred resistance showed the same MOI dependency as resistance in the homologous cell.

If transfer of resistance is dependent on cell proximity, then the degree of transfer should be controlled by both the donor (L) to receptor (WISH or BHK) cell ratio (shown in Table 1) as well as the absolute cell density

Table 2 Characteristics of the transfer of viral resistance

| Cells<br>( $2.25 \times 10^5$ cells/well) | % Inhibition of expected virus yield ( $P^*$ ) after treatment with:             |                                                    |                                                                            |                                                               |
|-------------------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------|----------------------------------------------------------------------------|---------------------------------------------------------------|
|                                           | Purified L cell interferon<br>( $100 \text{ PDD}_{50} \text{ U ml}^{-1}$ ) + VSV | Actinomycin D<br>( $5 \mu\text{g ml}^{-1}$ ) + VSV | C243 cell interferon ( $7,500 \text{ PDD}_{50} \text{ U ml}^{-1}$ )<br>VSV | Vaccinia virus<br>( $1 \times 10^4 \text{ p.f.u. per well}$ ) |
| L cell: WISH cell (1 : 1)                 | 90 (< 0.025)                                                                     | 0 (> 0.5)                                          | 78 (< 0.05)                                                                | 93.3 (< 0.01)                                                 |
| L cells                                   | > 99.98 (< 0.025)                                                                | 56 (> 0.2)                                         | 99.8 (< 0.025)                                                             | 98.5 (< 0.025)                                                |
| WISH cells                                | 0 (> 0.5)                                                                        | 0 (> 0.5)                                          | 0 (> 0.5)                                                                  | 8 (> 0.5)                                                     |

For experimental conditions see Table 1.

\*Statistical analysis was carried out using Students *t* test.

at a given ratio. To test the variable of cell concentration the effect of variation of cell density of a constant ratio of L and WISH cells was studied. Specifically, L and WISH cells each at a concentration of  $1.5 \times 10^6 \text{ cells ml}^{-1}$  were serially diluted in twofold steps. L cells at each dilution were mixed 1 : 1 with WISH cells of the same dilution and the cell mixtures were plated in the presence or absence of mouse interferon ( $7,500 \text{ PDD}_{50} \text{ U ml}^{-1}$ ). It was found that at an L cell-WISH cell ratio of 1 : 1, the demonstration of transfer of viral resistance was dependent on the absolute cell density (Fig. 1a). Interferon-induced transfer of viral resistance was not observed until the majority of cells were in close contact with neighbouring cells.

Since our initial experiments were carried out using very high levels of interferon ( $100\text{--}7,500 \text{ PDD}_{50} \text{ U ml}^{-1}$ ), it was important to determine if transfer of viral resistance could be demonstrated at more physiological concentrations. Mouse L cells were cultured in a 1 : 1 ratio with WISH cells (cell number per well =  $2.25 \times 10^5$ ) in the presence or absence (control) of different concentrations of mouse interferon or in its absence. Other controls consisted of an equivalent number of either cell type alone in the presence or absence of mouse interferon. Fig. 1b shows that transfer of viral resistance requires levels of interferon above  $5 \text{ PDD}_{50} \text{ U ml}^{-1}$ . Almost maximum transfer of attainable resistance can be demonstrated with  $15 \text{ PDD}_{50} \text{ U ml}^{-1}$  of mouse interferon indicating that in the experimental conditions there is a limit to the degree of resistance which can be transferred. These data show that whereas transfer of viral resistance to heterologous cells requires more interferon than development of resistance in homologous cells the process is, nonetheless, fairly efficient in terms of the concentrations of interferon required. The concentrations of interferon necessary for transfer are well within physiological limits.

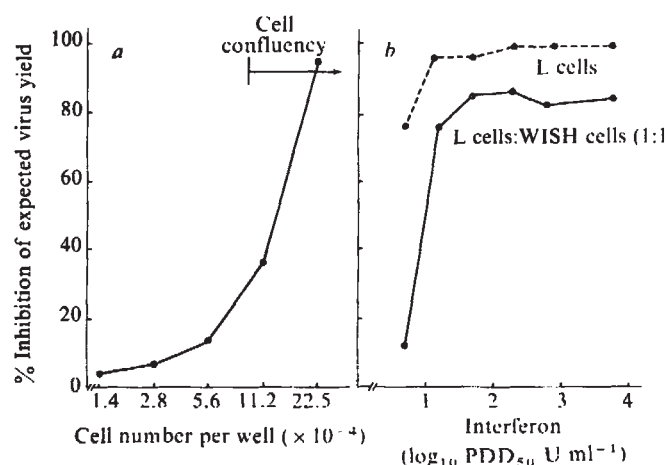
Although the exact mediator of the transfer of viral

resistance is unknown at present, it seems clear that it does not result from production of interferon by the heterologous cells in the mixture. The conclusion is based on two observations. First, interferon is thought to be externalised from a cell before acting<sup>16</sup>; however, in a mixture of 1 L : 1 WISH cell with mouse interferon, transfer of viral resistance occurred in the presence of antiserum to human fibroblast interferon (enough to neutralise  $300 \text{ PDD}_{50} \text{ U ml}^{-1}$  of any interferon which might be produced, data not shown). Second, transfer of viral resistance can occur from L cells to a line of African Green Monkey kidney cells (Vero) which have lost the ability to synthesise interferon (data not shown)<sup>17</sup>.

Another possibility is that human WISH cells are made sensitive to mouse interferon by co-cultivation with mouse L cells. Preliminary experiments indicate that this is probably not the case, since L cells treated with mouse interferon and washed extensively to remove the interferon can transfer resistance to WISH cells that are added after interferon have been removed (data not shown).

There are several implications of interferon-induced transfer of viral resistance between animal cells. First, some part(s) of the interferon system are apparently not 'species' specific because interferon-treated mouse L cells can transfer viral resistance to human WISH and baby hamster kidney cells. Second, the natural mechanism of interferon protection may include action on cells near the interferon-responding cell by means of this transfer mechanism. Such an idea has been proposed<sup>18</sup> to explain the shallowness of the slopes of interferon dose-response curves<sup>18</sup>. This type of secondary action would amplify the efficiency of the interferon system. Third, since cell lines differ in the rate at which they develop resistance in response to interferon, one might expect in conditions of transfer of resistance in mixed populations of cells as occurs *in vivo*, more slowly responding cells might be influenced by cells which respond more rapidly to interferon. In other words, the rate of development of interferon-induced resistance in a mixed population of cells would be determined by the first responding cell type. In fact, in carefully controlled kinetic studies human WISH cells treated with  $30 \text{ PDD}_{50} \text{ U ml}^{-1}$  human interferon developed significant resistance (50% inhibition of virus yield) to virus infection after 30 min treatment whereas human amnion U cells required 2.5 h to develop an equivalent degree (58% inhibition of virus yield) of resistance. When these two cell lines were mixed in a 1 : 1 ratio, the mixed population of cells developed significant viral resistance (63% inhibition of virus yield) after 30 min. That is, the mixed population assumed the kinetics of development of viral resistance of the more rapidly responding of the two cell types rather than an average (data not shown). This phenomenon would also tend to amplify the efficiency of the interferon system. Furthermore, this finding suggests that interferon-induced transfer of viral resistance occurs between cells of the same species and supports the notion of a function *in vivo* for transfer of viral resistance. A second example of homologous transfer of resistance is provided by our finding that interferon-treated L cells, which have been washed and treated with excess antiserum

Fig. 1 Effect of cell density (a) and interferon concentration (b) on transfer of viral resistance between L cells and WISH cells. Since interferon-treated L cells produce a negligible amount of virus, the percentage inhibition of expected virus yield is calculated by dividing the yield from an interferon-treated L cell-WISH cell mix (1 : 1) by half of the yield from a similar but untreated L cell WISH cell mix, times 100.





to mouse interferon, can transfer viral resistance to untreated L cells (data not shown).

A fourth implication is that interferon-induced transfer of viral resistance may represent a system for studying transfer of biological activity between cells. Finally, the interaction of interferon at the cell surface may cause the formation of a chemical intermediate which is responsible for induction of the antiviral protein. Such a chemical mediator might be passed from cell to cell with a resultant transfer of viral resistance. Preliminary experiments have indicated that cell-free supernatant fluids from interferon-treated L cells can transfer viral resistance to human WISH cells. The elucidation of the nature of the mediator of transfer of viral resistance would greatly enhance our understanding of the induction process for the antiviral state. Should this mediator prove to be a simple molecule, the potential for control of viral infections is obvious.

In summary, we have shown that interferon-treated mouse L cells can transfer their antiviral resistance to co-cultured heterologous (human WISH or hamster BHK) cells which are insensitive to mouse interferon. Transfer of viral resistance seems to be initiated by interferon itself. Once transferred, the viral resistance has the characteristics of the interferon-induced antiviral state. The transferred resistance occurs between several cell species. The transfer of resistance depends on the ratio of cells homologous (to interferon) to cells which are heterologous as well as to the absolute cell density at a given ratio. The transfer process is efficient in that it requires relatively small amounts of interferon. Finally, we propose that this phenomenon is a natural process for amplification of the interferon system, and preliminary evidence indicates that it occurs by cell to cell transfer of an interferon-induced molecule.

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complete Freund's adjuvant (CFA). It is generally regarded as an animal model of acute disseminated encephalomyelitis. In Lewis rats a hyperacute variant of EAE can be induced using appropriate CNS immunogen with *Bordetella pertussis* vaccine as adjuvant with or without CFA<sup>1-3</sup>. Hyperacute EAE (HEAE) differs from ordinary EAE clinically in its early onset and in its rapid and severe course, with high incidence of cerebral involvement and mortality. Histological lesions of HEAE are characterised by the presence in the CNS parenchyma of polymorphonuclear leukocytes, and necrosis, haemorrhage, oedema and fibrin<sup>1,2</sup>. Because HEAE and ordinary EAE overlap histopathologically and immunologically<sup>3</sup> they provide experimental evidence supporting the concept that certain human demyelinating diseases represent a spectrum of autoimmunity ranging from chronic multiple sclerosis to acute haemorrhagic necrotising leukoencephalopathy, for which HEAE is a model<sup>1,2,4</sup>. We report here that the induction of HEAE is favoured by the absence of a methyl group in a sequence of 14 amino acids of the primary structure of the immunogen.

The autoantigen in CNS tissue which induces and is the target of the immune response in EAE is the basic protein of myelin. Few differences are found in the sequences of the approximately 170 amino acids of mammalian basic proteins which have been characterised (reviewed in ref. 5). Numerous encephalitogenic determinants have been identified in the basic protein molecule (reviewed in ref. 6). In contrast to the similarities in amino acid composition of their basic proteins, different species vary markedly in their susceptibility to EAE induction by defined peptide regions of the basic protein. For example, the major encephalitogenic determinant for guinea pigs is the 'tryptophan' peptide (residues 113-121) (ref. 7). That peptide also induces EAE in rabbits<sup>8,9</sup>, but is inactive in rats<sup>9</sup>. Peptide 66-75 is encephalitogenic for rabbits<sup>10</sup>, but is inactive in rats<sup>11</sup>, and a peptide comprising residues 70-88 of guinea pig basic protein is known to be encephalitogenic for rats<sup>12-14</sup>. With *B. pertussis* as adjuvant, we found that guinea pig basic protein was unique in its capacity to induce HEAE in rats when compared with basic proteins from seven other species<sup>3</sup>. We further established that the determinant of the guinea pig basic protein responsible for HEAE induction resided in the region 70-88 (ref. 12). We have now confirmed by synthesis that the unique antigenicity of the guinea pig basic protein leading to HEAE induction is determined by the primary amino acid sequence of a 14 amino acid peptide. The corresponding region of the rat basic protein, which differs by only a single amino acid substitution at residue 79 (threonine/serine), induces ordinary EAE but not HEAE.

Guinea pig and rat basic proteins were prepared from brain tissues<sup>3</sup> and the corresponding midpeptides (residues 45-88) were isolated after 20 min of peptic digestion<sup>8</sup>. The midpeptides were further digested by rapid proteolysis with  $\alpha$ -chymotrypsin (20-30 s at 37 °C). Preparative electrophoresis in pyridine-acetic acid-butanol-H<sub>2</sub>O (1:1:2:36) yielded two fragments of 19 and 25 amino acids. Amino acid analyses identified the fragments as residues 70-88 and 45-69 respectively (Table 1). Peptide 73-86 (guinea pig sequence) was synthesised by the Merrifield solid phase technique.

Graded doses of basic proteins and peptides were prepared in saline, emulsified with CFA and injected intradermally into 10-week-old female Lewis rats as described previously<sup>3</sup>. *B. pertussis* vaccine ( $2 \times 10^{10}$  organisms) was injected subcutaneously. Clinical signs of ordinary EAE and HEAE were scored daily. Ordinary EAE generally appeared around day 12 or later and consisted of flaccid weakness or paralysis of limbs and/or tail, and incontinence, and was usually not fatal. HEAE was readily distinguished from ordinary EAE clinically by the additional combination

## Hyperacute autoimmune encephalomyelitis induced by a synthetic autoantigen

EXPERIMENTAL autoimmune encephalomyelitis (EAE) is an organ-specific autoimmune disease of the central nervous system (CNS) induced by immunisation with CNS tissue in

**Table 1** Encephalitogenicity of graded doses of guinea pig and rat myelin basic proteins and peptides of their midregions

| Immunogen                              | Dose (nmol)  | Mean day of onset | Clinical EAE<br>Total incidence of signs | Incidence of 'cerebral' signs | Mortality | Mean day of death |
|----------------------------------------|--------------|-------------------|------------------------------------------|-------------------------------|-----------|-------------------|
| Guinea pig basic protein               | 0.53         | 10                | 5/5                                      | 4/5                           | 5/5       | 12                |
|                                        | 0.17         | 10                | 5/5                                      | 4/5                           | 5/5       | 15                |
|                                        | 0.053        | 12                | 4/5                                      | 0/5                           | 0/5       | —                 |
| Rat basic protein                      | 0.21         | 16                | 4/5                                      | 0/5                           | 0/5       | —                 |
|                                        | 0.067        | —                 | 0/5                                      | 0/5                           | 0/5       | —                 |
| Guinea pig residues 45–88              | 1.0 –3.1     | 9                 | 24/24                                    | 20/24                         | 22/24     | 12                |
|                                        | 0.62         | 11                | 10/10                                    | 6/10                          | 4/10      | 17                |
|                                        | 0.062–0.21   | 12                | 8/10                                     | 0/10                          | 1/10      | 12                |
|                                        | 0.021        | —                 | 0/5                                      | 0/5                           | 0/5       | —                 |
| Rat residues 45–86                     | 0.64 –3.2    | 12                | 22/22                                    | 5/22                          | 2/22      | (12,21)           |
|                                        | 0.21         | 14                | 2/4                                      | 0/4                           | 0/4       | —                 |
|                                        | 0.021–0.064  | —                 | 0/7                                      | 0/7                           | 0/7       | —                 |
| Guinea pig residues 45–69*             | 0.55 –5.5    | —                 | 0/9                                      | —                             | 0/9       | —                 |
| Guinea pig residues 70–88†‡            | 0.16 –2.3    | 9                 | 33/33                                    | 22/33                         | 27/33     | 12                |
|                                        | 0.0046–0.037 | 13                | 13/14                                    | 4/14                          | 7/14      | 15                |
|                                        | 0.0028       | —                 | 0/4                                      | —                             | 0/4       | —                 |
| Rat residues 68–86†                    | 0.60 –2.3    | 11                | 25/25                                    | 15/25                         | 3/25      | 13                |
|                                        | 0.16         | 13                | 4/5                                      | 0/5                           | 0/15      | —                 |
|                                        | 0.018 –0.037 | —                 | 0/5                                      | —                             | 0/5       | —                 |
| Guinea pig residues 73–86§ (synthetic) | 1.2          | 10                | 12/12                                    | 11/12                         | 8/12      | 13                |
|                                        | 0.15         | 13                | 8/8                                      | 3/8                           | 3/8       | 18                |

HEAE was distinguished from ordinary EAE clinically by a combination of early onset (usually 9–10 d), rapid and severe course and high incidence of mortality and cerebral signs (irritability, seizures, coma and spasticity). Amino acid analyses were determined using a Beckman 121 analyser after hydrolysis for 24 h in 6 M HCl.

\*Amino acid analysis: Phe (1.0), Gly (3.0), Ser (3.0) Asp (2.0) Arg (3.0), Ala (4.0), Pro (1.0), Lys (2.0), His (3.0), Thr (2.0), Tyr (1.0).

†The rat sequence (68–86) is identical to the guinea pig sequence (70–88) except for a threonine for serine substitution.

‡70 88  
Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-[Ser]-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe  
(1.0)(3.0)(1.0)(2.0)(4.0)(1.0) (1.0) (2.0) (2.0) (1.0)(1.0)

§73 86  
Pro-Gln-Lys-Ser-Gln-Arg-[Ser]-Gln-Asp-Glu-Asn-Pro-Val-Val  
(2.0)(4.2)(1.0)(1.8) (1.0) (2.0) (1.8)

of early onset of signs (9–10 d), severe motor impairment with a high incidence of cerebral involvement (irritability, seizures, coma and spasticity) and a uniformly rapid and fatal course. Brains and spinal cords were examined histologically<sup>3</sup>.

Table 1 compares the encephalitogenicity of the whole guinea pig basic protein, its midpeptide (residues 45–88) and smaller fragments of that peptide. Doses as low as 0.17 nmol of guinea pig basic protein, 1.0 nmol of peptide 45–88 and 0.16 nmol of the chymotryptic fragment 70–88 induced HEAE. Ordinary EAE was induced by doses of peptide 70–88 as low as 0.0046 nmol. The larger chymo-

tryptic fragment 45–69 induced neither clinical nor histological signs of EAE at 5.5-nmol doses. The synthetic peptide comprising residues 73–86 induced HEAE (both clinical and histological signs (Fig. 1)) at a dose of 1.2 nmol.

Peptides corresponding to guinea pig residues 45–88 and 70–88, but derived from syngeneic rat basic protein, induced ordinary EAE, but not HEAE, when tested over a broad range of doses with identical adjuvants. Doses of 2.3 and 3.2 nmol induced very severe signs of ordinary EAE but cerebral signs were uncommon and most rats recovered. Thus the unique capacity of the guinea pig basic protein to induce HEAE (ref. 3) is not simply a dose effect but is determined by the amino acid sequence of the region 73–86. The only sequence difference in this region of the basic proteins of guinea pig (which induces HEAE) and rat (which induces only ordinary EAE even at high doses) is a serine/threonine substitution at residues 79. Thus the antigenic requirements for induction of two clinically distinct autoimmune syndromes are determined by one methyl group.

The fact that HEAE was induced by the synthetic peptide 73–86 rules out the possibility that the enzymatically derived guinea pig peptide might have been contaminated with another encephalitogenic fragment. Synthesis of additional analogues of the region comprised in residues 73–86 should allow definition of (1) the minimal antigenic requirements for induction of HEAE and (2) the essential difference between determinants inducing HEAE and ordinary EAE. The availability of defined synthetic autoantigens presents a unique opportunity to analyse the different immunopathogenic mechanisms involved in ordinary and hyperacute EAE.

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**Fig. 1** A small blood vessel with intraluminal inflammatory cells in the spinal cord parenchyma of a rat inoculated 12 d earlier with synthetic peptide 73–86 (Pro-Gln-Lys-Ser-Gln-Arg-[Ser]-Gln-Asp-Glu-Asn-Pro-Val-Val). The prominent extravasation of fibrin is characteristic of HEAE (phosphotungstic acid-haematoxylin,  $\times 779$ ).



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## Increased endoneurial fluid pressure in experimental lead neuropathy

THE endoneurial compartment of peripheral nerve is relatively inaccessible because of its small size and is maintained in a specialised environment by means of a perineurial barrier<sup>1</sup>, a blood–nerve barrier<sup>2</sup> and a cerebrospinal fluid barrier. We have gained access to the endoneurial compartment of mammalian peripheral nerve by means of small polyethylene matrix (PEM) capsules and have recorded endoneurial fluid pressure (EFP) using an active servo null system<sup>3</sup>. The solid PEM capsules have pores of approximately 60  $\mu$ m which facilitate entry of fluid into the interstices and connecting polyethylene tubing, but unlike hollow capsules<sup>4</sup>, are not invaded by connective tissue. We have used PEM capsules to make serial measurements of EFP in control and lead-fed rats and watched the pathological changes. We did this because of the prominence of endoneurial oedema in, for example, inflammatory polyradiculoneuropathy<sup>5</sup>, diabetic neuropathy<sup>6</sup>, acromegalic neuropathy<sup>7</sup> and experimental lead<sup>8</sup> and galactose<sup>9,10</sup> neuropathies, and because external pressure causes segmental demyelination and even axonal degeneration of peripheral nerve fibres<sup>11</sup>. We found that EFP increased progressively in lead-fed rats and preceded the onset of fibre pathology. The time course of the increase and of fibre pathology suggests that increased EFP is a cause of segmental demyelination in lead neuropathy.

PEM capsules (external diameter 0.5 mm) tightly wedged in a PE-10 tubing were implanted into the left sciatic nerve of 18 Sprague–Dawley rats and EFP recordings were made 1 month later. Pressures from the capsules were measured using a modification of an active servo-null device originally developed by Wiederhielm<sup>12</sup>. A micropipette containing hyperosmolar saline forms one arm of a balanced Wheatstone bridge adjusted for null. When the micropipette is wedged into a tubing connected to the capsule the EFP causes a shift in the fluid interface at the tip of the pipette, causing an error voltage. This voltage is amplified and applied to a bellows system which generates a counter-pressure within the pipette, restoring the fluid interface to its original position. The hydraulic pressure is measured with a transducer and recorded<sup>3</sup>.

After the initial recording nine rats were placed on a

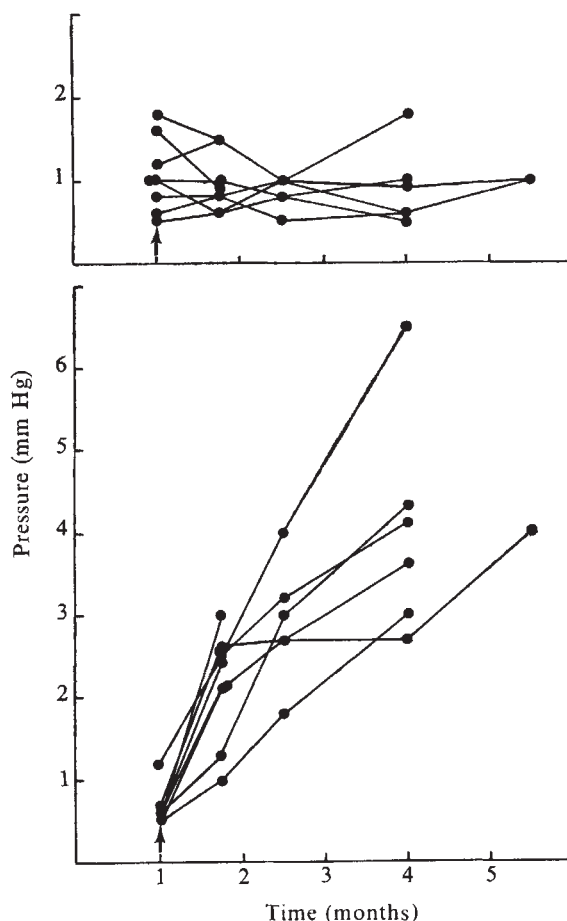
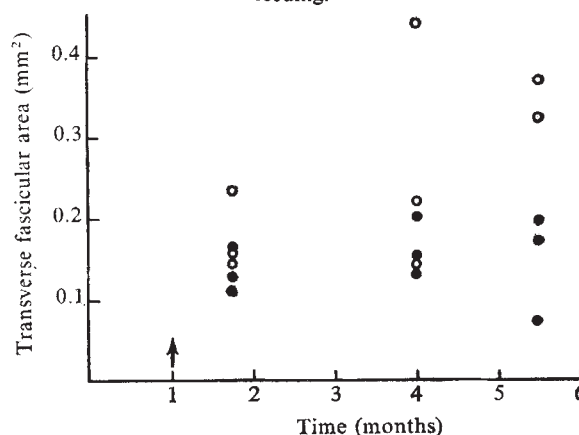


Fig. 1 EFP does not change significantly with time in control rats (upper) but increases progressively in lead rats (lower). Arrow indicates onset of test feeding.

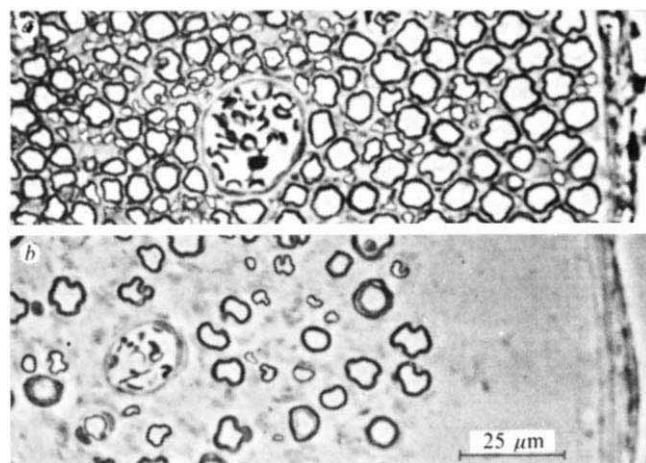
conventional diet to which 4% lead carbonate had been added (lead rats). The remaining nine animals were pair-fed the same diet but without the added lead (control rats). EFP recordings were repeated 3 weeks, 6 weeks, 3 months and 4.5 months after commencement of test feeding (Fig. 1). EFP recordings were successful in eight of nine control rats 1 month after implantation of the capsule and ranged from 0.5 to 1.8 mm Hg. They did not alter significantly in subsequent serial recordings. By contrast, the EFP was increased in seven of nine lead rats by 3 weeks after start of lead feeding and progressively increased in serial recordings (Fig. 1).

Three animals from each group were killed 3 weeks

Fig. 2 Transverse fascicular area of control (closed circles) and lead rats (open circles) at different times after start (arrow) of test feeding.







**Fig. 3** Transverse section of peroneal nerve from control (upper) and lead rat (lower) after 3 months of lead feeding. Note the marked pericapillary and subperineurial oedema in the nerve of the lead rat.

and 3 months after start of test feeding and the remaining three controls and two lead rats (one meanwhile had died) were killed at 4.5 months. All morphological studies were done on the right peroneal nerve.

The transverse fascicular area (TFA) of right peroneal nerve was digitised on photographic enlargements ( $\times 230$ ) of transverse sections of nerves embedded in Epon. The TFA was increased at 4½ months after test feeding and was variably increased even earlier in lead rats (Fig. 2). The number of measurements were small but the trend paralleled that of EFP recordings.

On pathological evaluation, endoneurial oedema was absent or mild at 3 weeks and prominent by 3 months. Oedema tended to be most pronounced around small blood vessels and in the subperineurial area (Fig. 3). Fibre pathology, as assessed by phase contrast microscopy of sections and by evaluation of teased fibres (100 fibres per nerve) consisted chiefly of segmental demyelination and remyelination. Axonal degeneration was uncommon. In Table 1 fibres showing segmental demyelination with or without regeneration are grouped under percentage demyelination. Segmental demyelination was not found in nerves of control rats or in nerves of rats fed lead for 3 weeks. At 3 months, however, segmental demyelination was seen in all lead rats and the changes were marked by 4½ months (Table 1).

Our study provides the first evidence that endoneurial pressure is increased in a neuropathy. The rise in EFP in experimental lead neuropathy is small in absolute terms and relative to the externally applied pressures which have been used to produce segmental demyelination<sup>11,13</sup>; however, the relative increase above baseline values is considerable. The

mean EFP after 6 weeks of lead feeding is approximately four times and at 3 months six times baseline values. Moreover, the mode of action of experimental compression is mechanical<sup>14</sup> and acute, compression being applied for minutes to an hour or two, whereas the pressure rise in experimental lead neuropathy as reported here is maintained over months. The magnitude of the increase in pressure is not sufficient to collapse endoneurial and perineurial capillaries<sup>15</sup> so that ischaemia from capillary collapse, as was postulated by Sunderland<sup>16</sup> for median nerve entrapment in man, should not be the mechanism of segmental demyelination in this model.

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## Development-related changes of triiodothyronine binding to brain cytosol receptors

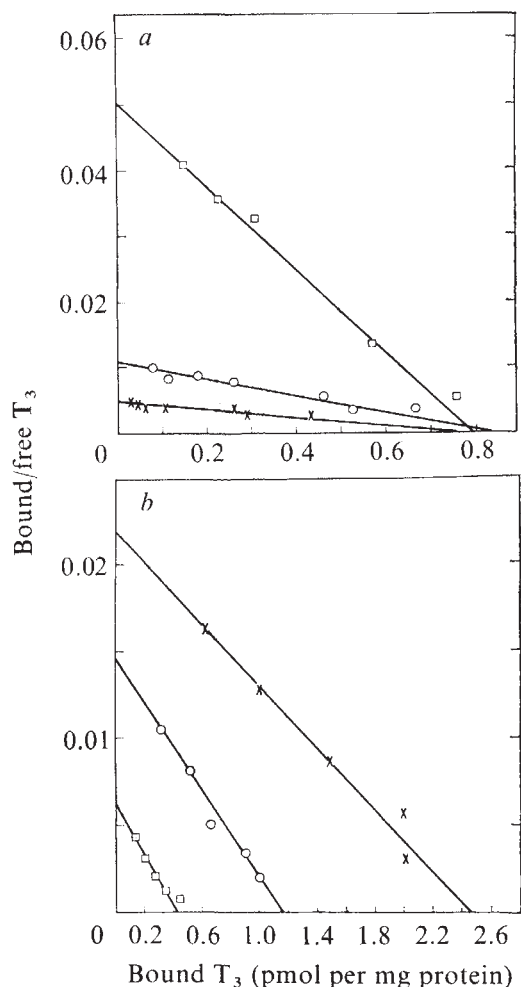
SEVERAL lines of evidence have established that thyroid hormones have a major role in the functional differentiation of the mammalian central nervous system<sup>1–4</sup>. Clinical or experimental hypothyroidism in early life results in behavioural disturbances and mental dysfunction which are apparently the outward manifestation of well-defined biochemical and structural lesions observed in the cerebellum and cerebral cortex of experimental animals. Significantly, the defects arising from the hypothyroid state are amenable to hormone therapy only during an early critical age period. Furthermore, unlike the liver and most other tissues, the mature brain is not responsive to the manipulation of the thyroid hormone state at least in terms of certain biochemical parameters. The brain is thus uniquely dependent on thyroid hormones for the full expression of functional development during a limited age period which is analogous to the situation in amphibian metamorphosis<sup>5</sup>. I reasoned that the acquisition and disappearance of brain sensitivity to thyroid hormones may be related to their binding properties with cellular receptors. I show here that the affinity of rat brain (cerebellum) cytosol for triiodothyronine ( $T_3$ ) is high during an early age period when cytodifferentiation is most intense and declines dramatically with age with no apparent change in the number of binding sites. In sharp contrast, the liver shows a marked increase in the number of binding sites with age with little change in affinity.

The rat cerebellum was chosen for study since both the structural and functional differentiation of this region at an early age and its responsiveness to the thyroid hormones

**Table 1** Percentage of teased fibres (of 100) showing segmental demyelination with a without remyelination

| Duration of study (weeks) | Nerve no. | Control | Lead |
|---------------------------|-----------|---------|------|
| 3                         | 1         | 0       | 0    |
|                           | 2         | 0       | 0    |
|                           | 3         | 0       | 0    |
| 12                        | 1         | 0       | 96   |
|                           | 2         | 0       | —*   |
|                           | 3         | 0       | —*   |
| 18                        | 1         | 0       | 94   |
|                           | 2         | 0       | 80   |
|                           | 3         | 0       |      |

\*Improperly prepared for adequate teased assessment—most fibres of both nerves showed segmental demyelination with or without remyelination.



**Fig. 1** Scatchard analysis of binding of  $^{125}\text{I-T}_3$  to cytosol prepared from rat (a) cerebellum and (b) liver at 10 ( $\square$ ), 20 ( $\circ$ ) and 50 ( $\times$ ) days of age. Cytosol (100–150  $\mu\text{g}$  protein) was incubated with increasing concentrations of  $^{125}\text{I-T}_3$  ( $4 \times 10^{-10}$  to  $1 \times 10^{-8}$  M, final concentration) for 2 h at  $0^\circ\text{C}$ . Bound hormone was separated from free hormone by the addition of 0.2 ml of a dextran-coated charcoal suspension (5% charcoal: 1% dextran of molecular weight 60,000–90,000 in 20 mM Tris-HCl, pH 7.8). The tubes were vortexed for 6 s, kept on ice for 10 min and centrifuged at  $4000g$  for 10 min. A portion of the clear supernatant containing the bound  $^{125}\text{I-T}_3$  was rapidly removed and counted in a Packard Auto-gamma spectrometer. Sephadex G25 chromatography<sup>21</sup> of the bound fraction confirmed that 94% of the radioactivity was in the form of  $\text{T}_3$ . The magnitude of the high affinity, saturable binding was estimated by subtraction of the radioactivity bound in the presence of a 1,000-fold molar excess of native  $\text{T}_3$ . The free hormone concentration was calculated by subtraction of the total  $^{125}\text{I-T}_3$  bound from the total  $^{125}\text{I-T}_3$  added per assay and expressed as nmol per l of cytosol. The dextran-coated charcoal procedure removed more than 98% of the  $^{125}\text{I-T}_3$  at all concentrations in the absence of cytosol. The removal of the bound hormone by Sephadex G25 gel filtration gave comparable results with the charcoal procedure. The values are means of two determinations which did not differ by more than 3% from the mean. The values are not corrected for the dissociation of the bound hormone in the presence of charcoal.

during this period have been extensively characterised. Cytosol was prepared from the cerebellum and liver (perfused through the portal vein with 25 mM  $\text{KH}_2\text{PO}_4$ , 0.1 M NaCl, pH 7.4) of 10-, 20- and 50-d old Sprague-Dawley rats. The tissues were homogenised with a motorised Teflon pestle in 5 volumes of 0.32 M sucrose, 20 mM Tris, 1.0 mM  $\text{MgCl}_2$  (pH 7.6). After removal of the mitochondrial fraction by centrifugation in the cold at  $12,000g$  for 20 min, the supernatant was centrifuged at  $130,000g$  for 90 min at  $4^\circ\text{C}$ . The supernatant, which represented the cytosol, was aspirated (excluding the surface lipid in the case of the

liver) and stored in small portions at  $-20^\circ\text{C}$  for no longer than 2 weeks. Cytosol, equivalent to 100–150  $\mu\text{g}$  protein was incubated in the presence of  $^{125}\text{I-T}_3$  (specific activity 290 Ci  $\text{mmol}^{-1}$ , Abbott) in  $12 \times 75\text{-mm}$  polypropylene tubes for 2 h at  $0^\circ\text{C}$  in a final volume of 0.5 ml. The reaction medium contained 0.25 M sucrose, 20 mM Tris, 1.0 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol and 5% glycerol (pH 7.6) and was used for the dilution of cytosol and labelled and unlabelled hormones. The presence of dithiothreitol was found to be necessary for maximal binding and glycerol was included to prevent receptor decay particularly over long incubation intervals. A second identical series of tubes contained, in addition, 1,000-fold molar excess of unlabelled  $\text{T}_3$  as a competitor for high affinity (saturable)  $^{125}\text{I-T}_3$  binding.  $^{125}\text{I-T}_3$  was at least 92% pure as determined by thin-layer chromatography. The level of cytosol protein as used in the assay was within the range where binding was directly proportional to protein for all age groups.

Representative Scatchard plots of the binding of increasing concentrations of  $^{125}\text{I-T}_3$  to cerebellar and liver cytosol during development are shown in Fig. 1a and b, respectively. Saturation was achieved with about  $1 \times 10^{-8}$  M  $\text{T}_3$  at all ages. The binding parameters, affinity constant and maximum binding capacity, derived from these curves are listed in Table 1. The striking decline in the affinity constant for the cerebellum with age apparently correlates with the limited period of responsiveness of the brain to thyroid hormones during and early critical period of development. In the liver, on the other hand, which does not demonstrate an age-dependent tissue sensitivity to thyroid hormones, the number of binding sites increases markedly with age with little change in the affinity constant.

The development of the cerebellar cortical architecture in the rat is almost exclusively confined to the first 3 weeks of life and the sequence of events have been clearly documented<sup>6</sup>. These maturational processes are dependent on the presence of thyroid hormones; thus, neonatal hypothyroidism retards cellular proliferation and differentiation and impairs the growth of neuronal processes, the formation of synapses and their organisation<sup>7–9</sup>. Physiological levels of hormone are capable of reversing these aberrant changes if administered at an early age<sup>10</sup>.

It is significant that the affinity constant for the cerebellum is high at an age when cellular proliferation and migration are maximal and cytodifferentiation is proceeding rapidly. This relationship implies that the regulation of gene expression may come under hormonal control. Evidence that thyroid hormones bind to nuclear receptors with high affinity in several tissues, including brain<sup>11–18</sup>, provides support for this contention.

The significance of recognition sites for  $\text{T}_3$  in brain cytosol shown here and earlier in several mammalian non-neuronal tissues<sup>13,19–23</sup>, is not clear. The  $\text{T}_3$  binding characteristics in cytosol seem to be considerably different from nuclear preparations<sup>17,19</sup>. Moreover, unlike steroid hormones, cytosol binding is apparently not a prerequisite for the interaction of thyroid hormones with nuclear re-

**Table 1** Association constants ( $K_a$ ) and maximal binding capacity ( $M$ ) of cerebellum and liver during development

| Tissue     | Age (d) | $K_a$ (l per mol $\times 10^{-7}$ ) | $M$ (pmol per mg protein) |
|------------|---------|-------------------------------------|---------------------------|
| Cerebellum | 10      | 6.40                                | 0.79                      |
|            | 20      | 1.25                                | 0.87                      |
|            | 50      | 0.66                                | 0.75                      |
| Liver      | 10      | 1.43                                | 0.43                      |
|            | 20      | 1.25                                | 1.16                      |
|            | 50      | 0.88                                | 2.45                      |

$K_a$  and  $M$  were derived from Fig. 1 where the slope represents  $-K_a$  and the intercept of the line in the abscissa equals  $M$ .



ceptors<sup>11,13,14,17</sup>. Cytosol binding sites may serve to provide a readily available supply of hormone for biological action at some additional intracellular site and thus may be regarded as 'acceptors'. Alternatively, cytosol binding sites may indeed be regarded as one of several primary receptors in accordance with the multiple actions of thyroid hormones.

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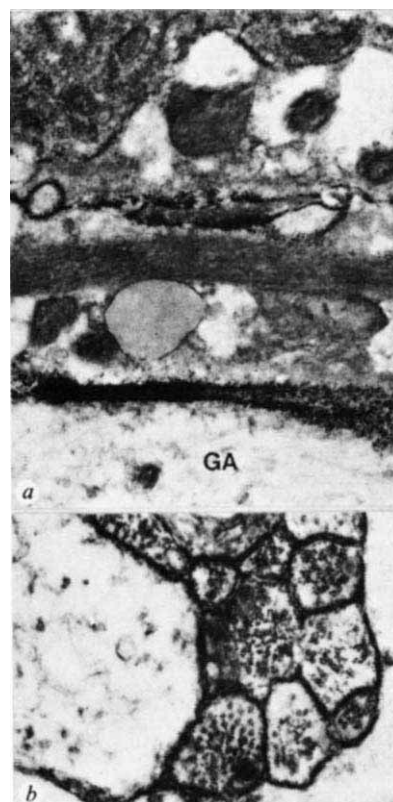
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## Axonal accessibility and adaptation to osmotic stress in an extreme osmoconformer

It is generally assumed that neuronal function is critically dependent on a relatively stable chemical environment. In most animals investigated homeostasis of the neuronal microenvironment is achieved by regulation of the chemical composition of the body fluids and, in higher vertebrates<sup>1–3</sup> and insects<sup>4</sup>, by additional homeostatic control within the nervous system. The axons of an extreme invertebrate osmoconformer<sup>5</sup> (the worm *Mercierella enigmatica* Fauvel) have been shown, however, to adapt and to continue to produce sodium-dependent action potentials when exposed to massive blood dilution<sup>6,7</sup>. It has also been suggested that short-term protection is provided by a facultative blood-brain barrier which enables the axons to adapt relatively slowly to hyposmotic stress<sup>7</sup>. We now report that the effects isosmotic and hyposmotic dilution of the surrounding medium on the giant axons of *Mercierella* are direct, and that axons respond rapidly to changes in ionic concentration and adapt to extreme osmotic stress almost immediately.

Electron microscopy showed that the giant axon is overlaid by narrow glial processes which form an incomplete investment. Where the covering is more complete the intercellular clefts which link the extra-axonal fluid with the



**Fig. 1** *a*, Giant axon (GA) and surrounding tissue of an animal adapted to seawater showing penetration of lanthanum through the glial clefts to the axon surface. Animals were incubated for 30 min in normal saline with 10 mM  $\text{LaCl}_3$  added before fixation by the method devised by Baskin<sup>8</sup> ( $\times 20,500$ ). *b*, Small axons from the connective of a seawater-adapted animal exposed briefly to dilute saline, a treatment previously supposed to limit access to the axon surface. Lanthanum is shown here to penetrate to the intercellular spaces between the axons. Animals briefly exposed to dilute saline were incubated in 50 mM  $\text{LaCl}_3$  for 2 h before fixation with glutaraldehyde in dilute saline, enriched with phosphate to a final concentration of 0.05 M and with added calcium. The preparation was post-osmicated and stained *en bloc* with uranyl acetate ( $\times 31,000$ ).

blood, or bathing medium, do not seem to be occluded by junctional complexes.

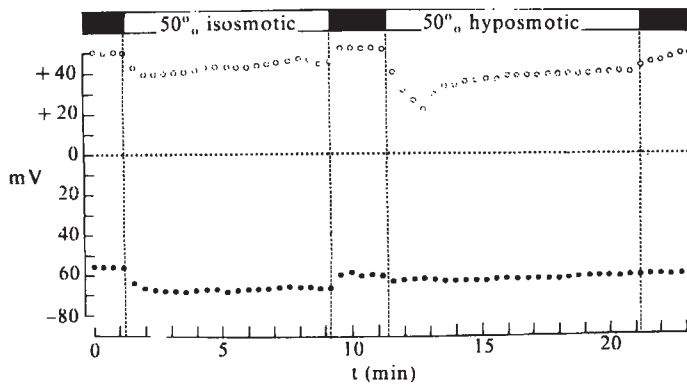
Externally-applied lanthanum was found to penetrate to the axon surfaces in seawater-adapted individuals bathed in normal (Fig. 1*a*) and diluted (10%) physiological saline (Fig. 1*b*). There is therefore no ultrastructural evidence for a significant restriction of intercellular diffusion to the axon surface in hyposmotic conditions.

We also found that the axonal responses to hyposmotic solutions depend on the method of dilution of the fluid in the recording chamber. With continuously flowing saline, as used before<sup>7</sup>, the lack of effect of rapid hyposmotic dilution resulted from fluid stratification which produced an unstirred layer of relatively high salinity in the immediate vicinity of the axon. When hyposmotic dilution was achieved by vigorously syringing the solution directly on to the preparation, relatively rapid axonal responses to ionic dilution could be observed in isosmotic and hyposmotic conditions (Fig. 2).

Our results indicate that the giant axon of *Mercierella* is not structurally protected from the immediate effects of hyposmotic stress. Even if some protection is provided in the intact animal by fluid stratification in the immediate vicinity of the axon surface, the giant axon can adapt to hyposmotic stress in experimental conditions much more rapidly than has been previously supposed<sup>7</sup>.

Intact colonies of *Mercierella* can function normally in distilled water for several weeks. Fig. 3*b* and *c* illustrates the

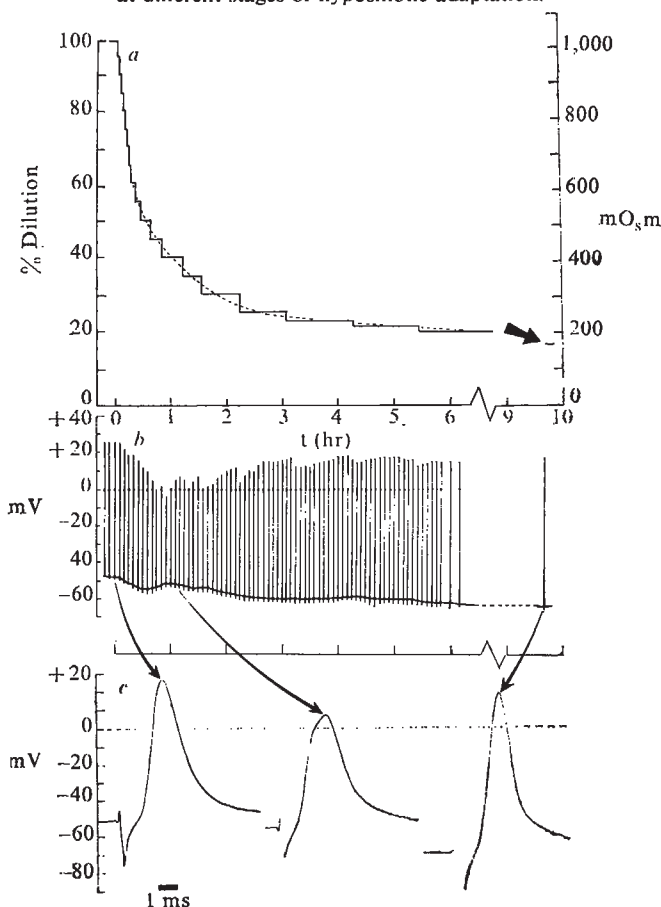




**Fig. 2** Effects of 50% dilution of the physiological saline (1,024 mOsm) on the action and resting potentials, recorded with a conventional intracellular microelectrode (40 M $\Omega$ ), in a giant axon of *Mercierella*. Hyposmotic saline was made by diluting standard saline with distilled water. Isosmotic dilution was achieved by addition of mannitol to diluted standard saline. In these experiments the solutions were syringed (at a rate of 0.5 ml s<sup>-1</sup>) directly on to the preparation. The standard saline was based on the ionic and osmotic concentrations of seawater-adapted animals (mM: Na<sup>+</sup>, 482.3; K<sup>+</sup>, 30; Mg<sup>2+</sup>, 77; Ca<sup>2+</sup>, 31; SO<sub>4</sub><sup>2-</sup>, 26; Cl<sup>-</sup>, 663.8; OH<sup>-</sup>, 12.5 and PIPES, 7.5 mM).

responses of the giant axon to a dilution regime (Fig. 3a) similar to that in the blood of intact animals after transfer of a colony from seawater to distilled water. In spite of extreme osmotic stress, action potentials persisted throughout the experiment, with some diminution in amplitude only during the initial period of dilution. The hyposmotically-

**Fig. 3** Effects of progressive hyposmotic dilution on the resting and action potentials in a giant axon of *Mercierella*. *a*, The broken line illustrates the rate of dilution of the blood measured on transfer of colonies of *Mercierella* from seawater to distilled water<sup>5</sup>. The continuous line indicates the step changes in dilution used in this experiment. *b*, Continuous recording of the resting and action potentials recorded by a transient signal processor and displayed on a chart recorder. *c*, Action potentials at different stages of hyposmotic adaptation.



adapted action potentials were of larger amplitude than those recorded initially in full strength saline. As shown from axons from individuals which were hyposmotically adapted in distilled water for several weeks<sup>7</sup>, the *in vitro* adaptation involves a relatively slow hyperpolarisation of the axon membrane. This, together with an apparent reduction in intracellular sodium concentration<sup>7</sup>, facilitates overshooting action potentials of large amplitude in the face of the extreme dilution of the ionic and osmotic concentration of the bathing medium.

These observations indicate that the giant axons in this euryhaline osmoconformer can withstand and adapt to extreme changes in the osmotic and ionic composition of its immediate fluid environment. This establishes the important principle that neuronal homeostasis can be achieved not only by regulation of the extracellular fluid (as in higher vertebrates and insects) but also by relatively rapid adaptations by the nerve cells themselves.

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## Modification of synaptic input following unilateral labyrinthectomy

UNILATERAL vestibular nerve transection is followed by postural and locomotor disturbances that disappear to a large extent with time after the lesion<sup>1</sup>. This compensation requires a central readjustment of the tonic and phasic output from the remaining vestibular apparatus to the motor nuclei involved in the control of limb and head muscles. To study the mechanisms of this type of motor learning at the single neurone level, we have used the frog (*Rana temporaria*), because in this species the time course of the postural compensation following hemilabyrinthectomy has been thoroughly studied<sup>2</sup>. Furthermore, the synaptic circuitry and functional organisation of the peripheral and central vestibular systems are well known for this species<sup>3</sup>. Since the vestibular nuclei are the first integrative structures in which bilateral vestibular and other sensory inputs converge, and since their output influences motor systems directly or indirectly, we began to look for plastic changes in second order vestibular neurones. Many of these neurones are monosynaptically excited from the ipsilateral<sup>4</sup> and disynaptically through commissural fibres from the contralateral<sup>5</sup> labyrinth.

After unilateral labyrinthectomy via a ventral, extracranial approach, acutely ( $\leq 12$  h) and chronically ( $\geq 60$  d) deafferented vestibular neurones were activated by electrical stimulation of the anterior branch of the intact contralateral VIIIth nerve. Dissection, stimulation, and recording procedures were as described previously<sup>4,5</sup>. Synaptic potentials were averaged with a Nicolet computer and corrected for the extracellularly recorded field potential<sup>6</sup>. Duration of excitatory postsynaptic potentials (e.p.s.ps) were measured at half amplitudes and the times-to-peak from the points of divergence of the intra- and extracellular records to the peak of the e.p.s.ps. To facilitate comparison of amplitudes, threshold values, and shape indices

of e.p.s.ps<sup>7</sup> amongst different animals, the stimulation intensity was expressed in multiples of the  $N_1$  field potential threshold ( $T_{N_1}$ ) recorded in the ipsilateral vestibular nucleus<sup>4</sup> and was restricted to  $\leq 5 \times T_{N_1}$ .

In acutely operated animals stimulation of the intact VIIIth nerve elicited field potentials in the vestibular nuclei on both sides (Fig. 1A) as described previously<sup>4</sup>. The presence of these field potentials served as a convenient measure of electrode location and the state of the animal.

Intracellular recordings from vestibular neurones located ipsi- and contralaterally to the stimulation side revealed e.p.s.ps with significant differences in their shape indices (Fig. 1B). The e.p.s.ps elicited from primary afferent fibres had a much greater amplitude ( $2.6 \pm 1.0$  mV;  $n = 45$ ) and a much shorter time-to-peak ( $4.2 \pm 1.6$  ms; Fig. 2) than those elicited through commissural fibres ( $1.6 \pm 1.1$  mV;  $P < 0.001$  and  $12.4 \pm 3.2$  ms;  $P < 0.001$ ;  $n = 75$ ; Fig. 2). With increasing stimulus intensity, the time-to-peak of the e.p.s.ps did not shorten systematically. The duration of the e.p.s.ps was in general about three times the time-to-peak but was often much longer because of the arrival of late polysynaptic potentials. The e.p.s.ps induced by primary afferent fibres regularly triggered bursts of partial<sup>4</sup> and full action potentials whereas e.p.s.ps induced by commissural fibres elicited single spikes in only 22.1% and repetitive firing in only 2.6% of the cells. The slower time-to-peak and the lower efficacy of commissural e.p.s.ps to provoke action potentials fully corroborate the suggestion<sup>5</sup> that commissural fibres form synapses more peripherally on the dendrites of vestibular neurones than primary afferent fibres which are known to synapse on and close to the soma<sup>8</sup>.

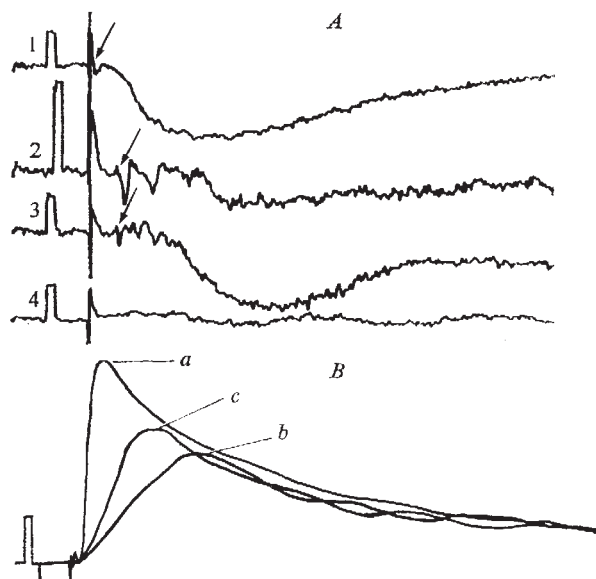
In chronic animals the vestibular-evoked field potentials

recorded in the deafferented vestibular nucleus (Fig. 1A) were of larger amplitude (two to three times) than those recorded in acute animals. Usually bursts of evoked spikes were superimposed on the field potential, a finding which was rarely observed in intact<sup>5</sup> or in acute animals from this series of experiments. In chronic preparations, in contrast to acute animals, many vestibular neurones were spontaneously active with firing rates (1–10 Hz) similar to those recorded on the intact side.

The increased excitability of deafferented vestibular neurones might have been caused by a change in the excitability of vestibular neurones located on the intact side which project to the deafferented nucleus. To investigate this possibility, we recorded extra- and intra-axonally from commissural fibres on the lesioned side of acute and chronic animals as well as in the depth of the brainstem where these fibres are known to cross<sup>9</sup>. A comparison of the data obtained in these two groups of experimental animals showed practically identical values for the thresholds of single spikes ( $1.6 \times T_{N_1}$ ), bursts ( $2.3 \times T_{N_1}$ ) and for the number of spikes per burst (2.9) as well as for the interspike interval (2.4 ms). These findings indicate that the excitability and the output pattern of vestibular neurones on the intact side have not changed postoperatively.

Intracellular recordings from vestibular neurones on the chronically deafferented side (Fig. 1B) showed that they receive predominantly excitatory inputs from vestibular commissural fibres as in intact animals<sup>5</sup>. The major characteristics of commissurally evoked e.p.s.ps from acute and chronic animals are compared in Table 1. As can be seen, e.p.s.ps in both groups of animals had similar thresholds and latencies. In chronic frogs, however, the amplitude of the evoked e.p.s.ps was significantly larger, and generated in many more cells partial and full action potentials at a significantly lower stimulus intensity than in acutely operated or intact animals<sup>5</sup>. The rise time of the e.p.s.ps was also significantly shorter in chronic than in acute animals (Fig. 2) and varied only a little ( $\pm 1.5$  ms) with increasing stimulus intensity. The duration of the e.p.s.ps varied greatly, but many of the e.p.s.ps recorded in chronic animals with a shorter time-to-peak had a longer duration. In addition polysynaptic components of the e.p.s.ps could prolong the falling phase considerably.

The changes in e.p.s.p shape indices and spike thresholds were observed in chronically deafferented neurones in spite of the absence of changes in threshold and bursting pattern of commissural input fibres. This indicates that the higher synaptic efficacy of the crossed excitatory input must be produced on the deafferented side somewhere between commissural terminals and postsynaptic neurones. One possible mechanism that could explain all of the observed changes in e.p.s.p. parameters would be axonal sprouting of commissural terminals resulting in the formation of new functional synapses on the vacated space on soma and proximal dendrites of vestibular neurones. As a result of the cable properties of dendrites<sup>7</sup>, these mixed den-



**Fig. 1** A, Field potentials in the vestibular nucleus recorded on the ipsilateral sides (1) and contralateral (2, 3, 4) to VIIIth nerve stimulation from an acutely (2) and a chronically (1, 3, 4) operated animal. The presynaptic components (arrows) indicate the arrival of impulses in primary afferent (1) and in commissural fibres (2, 3) respectively. Cutting the vestibular commissure abolished both the prepotentials as well as the later slow negative field potentials (4) recorded previously at the same depth (500  $\mu$ m; 3) but left the ipsilateral field potential (1) unchanged. The depth profiles of the slow negative field potentials of acute and chronic animals were similar (maximal response 450–500  $\mu$ m), but their amplitudes were different (compare 2 with 3). Calibration pulse: 0.5 mV; 1 ms; positivity upwards. B, E.p.s.ps recorded in vestibular neurones induced by primary afferent fibres (a) and by commissural fibres (b, c). b, was recorded in an acutely and c in a chronically deafferented neurone. Note the shorter time-to-peak and the larger amplitude of c in comparison with b. Traces are redrawn from the computer-averaged e.p.s.ps (16 sweeps) and were superimposed at the point of their onset. Downward artefacts represent stimulation; calibration pulse: +1 mV; 1 ms.

**Table 1** Excitation of deafferented second-order vestibular neurones

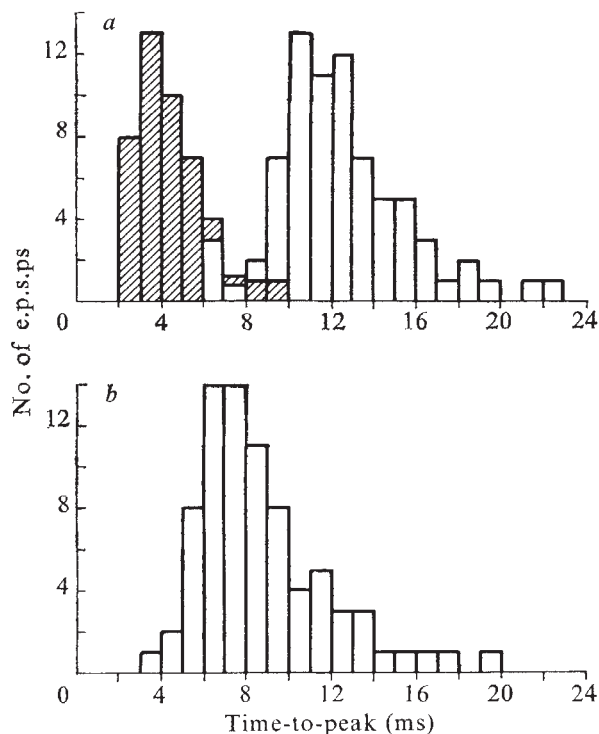
| Time after lesion                               | $\leq 12$ h    | $\geq 60$ d           |
|-------------------------------------------------|----------------|-----------------------|
| Number of e.p.s.ps                              | 75             | 78                    |
| $T_{e.p.s.p.}$                                  | $1.8 \pm 0.8$  | $1.6 \pm 0.5$         |
| Latency*                                        | $5.5 \pm 1.5$  | $5.8 \pm 1.6$         |
| Time to peak*                                   | $12.4 \pm 3.2$ | $8.6 \pm 3.1^\dagger$ |
| Amplitude*                                      | $1.6 \pm 1.1$  | $2.1 \pm 0.9^\dagger$ |
| Partial spikes in % of cells                    | 49.3           | 87.5                  |
| $T_{spike \leq 5 \times T_{N_1}}$ in % of cells | 22.1           | 55.1                  |
| $T_{spike \leq 5 \times T_{N_1}}$               | $3.4 \pm 1.0$  | $2.6 \pm 1.1^\dagger$ |
| $T_{burst \leq 5 \times T_{N_1}}$ in % of cells | 2.6            | 46.4                  |

T refers to the threshold for the evoked  $N_1$ -potential on the stimulated side.

\*Mean values  $\pm$  s.d.

$^\dagger$ Significantly different ( $P < 0.01$ ) from  $\leq 12$  h.

$^\ddagger$ Significantly different ( $P < 0.001$ ) from  $\leq 12$  h.



**Fig. 2** Distribution of times-to-peak of e.p.s.ps in acutely (a) and chronically (b) deafferented second-order vestibular neurones. The e.p.s.ps elicited from primary afferent fibres (cross-hatched bars) have a much shorter rise time than those elicited from commissural fibres. After chronic deafferentation the same commissural input induces e.p.s.ps with a significantly shorter rise time (b).

drift-somatic e.p.s.ps would be less attenuated in their rise times and amplitudes and, therefore, more effective in driving the cell. Similar changes in the shape indices of e.p.s.ps have been observed in other partially deafferented nuclei<sup>10</sup> where sprouting was shown to occur<sup>11</sup>. Another mechanism, for example, increased chemosensitivity of the denervated neurones to a given transmitter, is unlikely since the time-to-peak of the response does not seem to be affected<sup>12</sup>. Also, neither the ipsilateral nor the contralateral group of e.p.s.ps showed systematic shortening of the time-to-peak with increasing stimulus intensity. Changes in passive membrane properties, although not excluded, cannot explain all the alterations observed.

The increase in excitatory synaptic efficacy of the commissural system may provide part of the background activation of chronically deafferented vestibular neurones necessary to counterbalance the asymmetry in the output of the two vestibular nuclei at rest. As a result of this newly acquired symmetrical output, the animals will resume their normal pose. Furthermore, the enhanced crossed excitation will also be of use for dynamic postural reflexes requiring a symmetrical output to somatic motoneurones. To obtain functional recovery of all vestibular reflexes, crossed inhibitory mechanisms must also be postulated<sup>13</sup>; these will be subject of a later report.

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## Enkephalin effects on release of brain acetylcholine

MORPHINE and other narcotic analgesics depress the spontaneous release of acetylcholine (ACh) from the rat cerebral cortex *in vivo*<sup>1,2</sup>. The effect of these agents on the central release of ACh, like their inhibitory effects on the peripheral release of ACh in the guinea pig ileum, is stereospecific<sup>3</sup> and can be fully antagonised by naloxone<sup>4</sup>. It is well recognised that the endogenous opiate peptides, enkephalins<sup>5,6</sup> and endorphins<sup>7</sup>, depress the output of ACh from the electrically stimulated guinea pig ileum by a specific action on opiate receptors. This suggests that in the central nervous system endogenous opiates may exert a significant influence on the release of ACh. We have examined the effects of two enkephalins, methionine-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin) on the cortical release of ACh *in vivo* following intraventricular injections. Our observations show that natural enkephalins may interact with opiate receptors in the brain to modulate the release of ACh from the cholinergic neurones.

Experiments were carried out on Sprague-Dawley rats (250–300 g), lightly anaesthetised with a pentobarbital-urethane mixture. The spontaneous release of cortical ACh, which occurs in the presence of neostigmine (50  $\mu\text{g ml}^{-1}$ ), and atropine (0.5  $\mu\text{g ml}^{-1}$ ), was measured using the cup technique of MacIntosh and Oborin<sup>8</sup>. Experimental details of this procedure in rats, and the estimation of ACh, have been described elsewhere<sup>2,4</sup>. In this study, the collection periods for cortical ACh release were shortened to 10 min from the 20–30 min used in earlier tests. The samples of cortical fluid (0.2 ml each) were collected bilaterally and both samples were pooled before estimation of ACh. The peptides, in most experiments, were administered by intraventricular (i.v.t.) injection through a fine cannula placed in the lateral ventricle on the left side. In a few tests with Met-enkephalin, the intravenous (i.v.) route of administration was used. Naloxone was always administered by i.v. injection through a femoral catheter.

Met-enkephalin (20  $\mu\text{g}$ ) depressed the release of ACh during the first 10-min collection period after a single i.v.t. injection. Recovery of release to pre-injection levels occurred after about 50 min. When administered intravenously Met-enkephalin (2.5 mg per kg) was completely ineffective. In subsequent experiments effects of both Met-enkephalin and Leu-enkephalin on ACh release were investigated, and in these tests naloxone was administered to animals during the peptide-induced response. As shown in Fig. 1a and b, both enkephalins depressed ACh release to the same degree and their effect was fully reversed by naloxone. When administered before enkephalins, naloxone completely blocked the inhibitory effect of enkephalins on ACh release (Fig. 1c and d). In previous work with morphine, both depressant and stimulant effects on central ACh release have been reported<sup>3,9,10</sup>. In some cases low doses of opiates depress central ACh release while higher doses enhance this release<sup>3,10</sup>. To observe if enkephalin has a dual action on release, effects of several doses of Met-enkephalin on cortical ACh release were tested. Met-enkephalin produced only a dose-related inhibition of ACh release (Fig. 2). The inhibition produced by lower doses of

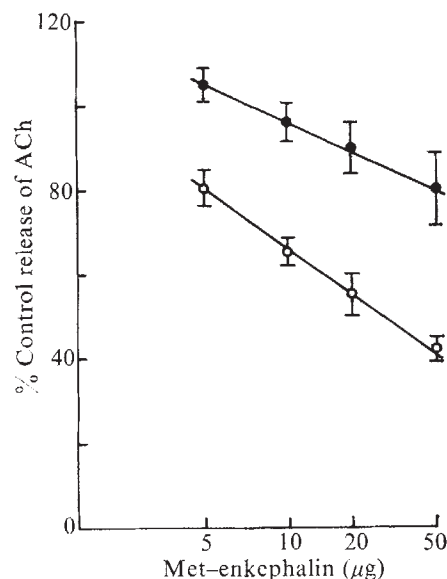
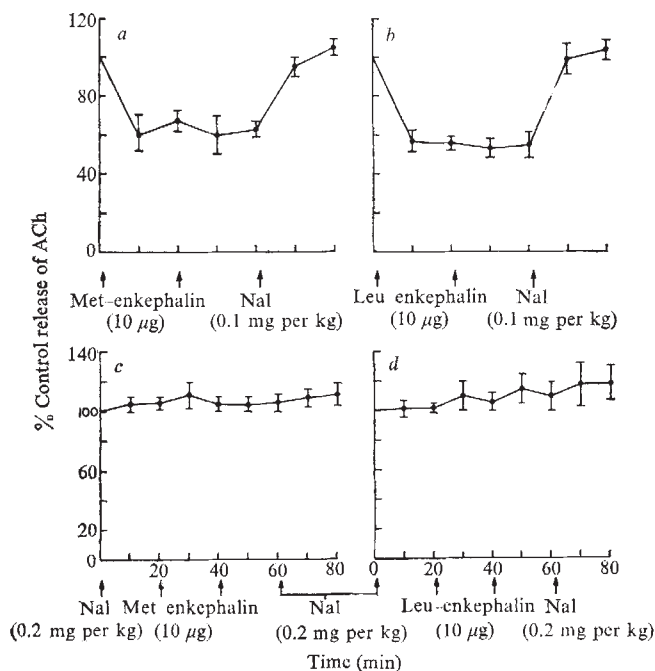


Met-enkephalin was fully reversed by naloxone when it was injected after the peptide, but the inhibition produced by larger doses was only partially reversed by the antagonist (Fig. 2).

Our findings show that both Met-enkephalin and Leu-enkephalin inhibit the spontaneous release of ACh from the cerebral cortex *in vivo*. The fact that their effect could be antagonised by the specific opiate antagonist naloxone suggests that these enkephalins are acting selectively on opiate receptors to influence the release of cortical ACh. In the guinea pig ileum<sup>8</sup>, and the opiate receptor binding assay<sup>11</sup>, Met-enkephalin is twice as potent as Leu-enkephalin. As only one dose (10 µg) of Leu-enkephalin was tested here, no definite conclusion can be drawn regarding the potency of action of two enkephalins on the central ACh release. At this dose, however, the effects of Met-enkephalin and Leu-enkephalin on ACh release were very similar and their action was completely blocked by naloxone. In previous work with opiate analgesics we have observed that when naloxone is administered after morphine-induced depression of ACh release, it produces a rebound effect on this release<sup>4</sup>. The post-naloxone level of output in such tests greatly exceeds the pre-drug control level<sup>3,4</sup>. Interestingly, no rebound effect could be observed when naloxone was given after the enkephalin induced depression. Furthermore, we did not observe the enhancement of ACh release by opiates which has been reported in certain circumstances<sup>3,9,10</sup>. Further investigation is necessary to clarify the basis for differences between the action of opiate analgesics and opiate peptides. In this regard the use of more stable analogues of enkephalins, endorphins or agents which inhibit the degradation of natural enkephalins may prove useful.

Naloxone administered by itself enhances the electrically

**Fig. 1** Inhibitory effect of (a), Met-enkephalin and (b), Leu-enkephalin on the release of ACh from the intact rat cerebral cortex. The antagonism of the effect of (c) Met-enkephalin and (d) Leu-enkephalin by pretreatment with naloxone (Nal). Enkephalins were administered by intraventricular injection in a 10 µl volume, and naloxone was given intravenously in all experiments. Control release represents average release of ACh during two collections preceding the first injection. Mean  $\pm$  s.e.m. of 3–6 experiments is shown.



**Fig. 2** Inhibition of cortical ACh release by several doses of Met-enkephalin, and the reversal of this inhibition by naloxone. ○, represents the release of ACh 10 min after intraventricular injection of Met-enkephalin; ●, represents release of ACh 10 min after intravenous injection of naloxone in the same animal. A separate group of animals was used for each dose of Met-enkephalin. Mean  $\pm$  s.e.m. of 3–4 experiments is shown.

stimulated release of ACh from the rat cortex<sup>4</sup>, and it has been suggested that this facilitatory effect of naloxone may be due to the antagonism of an endogenous opiate released by stimulation. This suggestion is strengthened by this demonstration of an inhibitory action of enkephalins on ACh release. Other investigators have reported an inhibition of noradrenaline<sup>12</sup> and dopamine<sup>13</sup> release from brain slices by endogenous opioid peptides. In view of these reports, and present findings in an *in vivo* model of ACh release, an attractive suggestion is that the enkephalins or endorphins in the central nervous system may serve to modulate the release of neurotransmitters resulting from activity in the catecholaminergic and cholinergic pathways.

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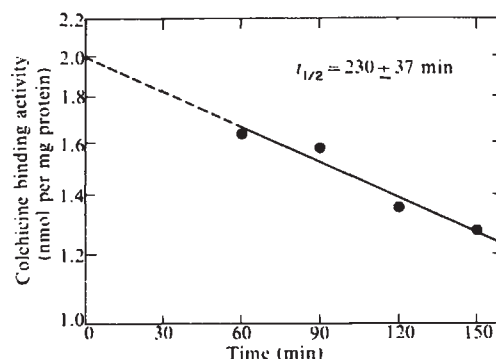
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## Polymerising ability of C<sub>6</sub> glial cell microtubule protein decays much faster than its colchicine-binding activity

BECAUSE of its high degree of specificity<sup>1</sup>, the binding of colchicine to tubulin has become a powerful tool for the quantification and subcellular localisation of the protein. The mechanism of the binding reaction is not yet completely understood, but several of its characteristics are well documented. One of these is the decay of binding capacity with time, which follows the kinetics of a first-order reaction<sup>2,3</sup> and is accentuated at elevated temperatures<sup>4</sup>. Although correlation between this rate of decay and the loss of other characteristic functions of tubulin, such as its ability to polymerise into microtubules, has not been directly established by experiment, carefully controlled measurements of the decay of colchicine binding seem to be useful for estimating the stability of the tubulin molecules *per se*. In contrast, the decay rate of the polymerisability of tubulin preparations should depend not only on the stability of the tubulin molecules themselves but also on that of other factors that may be essential for polymerisation<sup>5-7</sup>. As part of our recently initiated<sup>8,9</sup> attempt to study the mechanisms regulating microtubule assembly in cultured cells, we report here that tubulin preparations from rat glial C<sub>6</sub> cells exhibit a decay rate of colchicine-binding activity that is quite different from the decay rate of polymerisability.

Tubulin preparations purified from crude cell extracts by one cycle of *in vitro* polymerisation (with 4 M glycerol) and depolymerisation<sup>8,10</sup>, which by SDS-gel electrophoresis scans contained about 70% tubulin, were used in time course studies of both the capacity to bind colchicine and the ability to polymerise. The depolymerisation was carried out by suspending the tubulin pellet in glycerol-free buffer, since the stabilising effect of this reagent on polymerisability<sup>10</sup> and possibly on colchicine-binding activity<sup>11</sup>, might have obscured the results. The decay rates of colchicine-binding activity were determined by pre-incubation of sample aliquots at 37 °C for various lengths of time before assaying the binding of <sup>3</sup>H-colchicine<sup>1</sup>. The results of such an experiment, carried out with purified C<sub>6</sub> tubulin, are shown in Fig. 1. Typically, the binding activity decayed in an apparent first-order manner with a half life of 230 ± 37 min. In the same conditions, hog brain tubulin (three polymerisation cycles) had a half life of 293 ± 20 min. Purification of the C<sub>6</sub> tubulin by repeated cycles of polymerisation had no effect, within experimental error, on the kinetics of decay.

In other studies (G.W., L.S.H. and R.D.C., unpublished), it was found that *in vitro* microtubule formation from depolymerised C<sub>6</sub> tubulin is very similar to that of hog brain tubulin in that alike polymeric intermediates in the form of rings and planar and twisted ribbons are observed. The occurrence of such structures during the assembly of microtubules from hog brain tubulin has been studied extensively<sup>12-14</sup>. The decay rate of the polymerisability of depolymerised C<sub>6</sub> tubulin preparations may be determined directly, by measuring from electron micrographs<sup>12</sup> the total amount of polymer, that is, ribbons and tubules, that formed after incubation at 37 °C after various time intervals, following the last cold spin, at 0 °C. Monitoring assembly by quantitative electron microscopy has the advantage of allowing a distinction to be made between these ordered tubulin polymers and amorphous tubulin aggregates which affect measurements made by light scattering or viscometry. Throughout this study the incubation periods at 37 °C extended beyond the time during which ring structures could still be observed. The

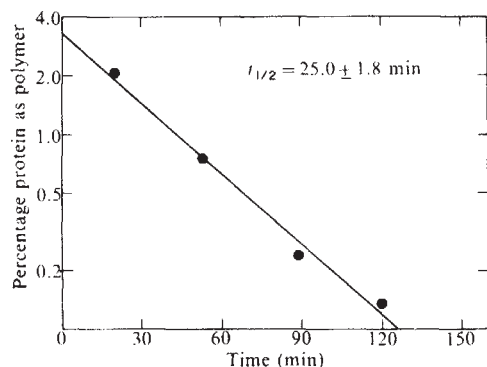


**Fig. 1** Decay rate of colchicine-binding activity.  $t_{1/2} = 230 \pm 37$  min. Rat glial cells (clone C<sub>6</sub>)<sup>8</sup> were grown suspended in Minimal Essential Medium (Joklik-Modified, No. F-13, Grand Island Biological, Grand Island, New York), supplemented with 5% calf serum, to a final density of approximately  $1 \times 10^6$  cells ml<sup>-1</sup>, and collected by centrifugation at 700g for 10 min at room temperature. Tubulin was purified from the packed cells by a modification of the Shelanski method<sup>10</sup> of *in vitro* polymerisation (but only one cycle) as described previously<sup>8</sup>. Pellets of polymerised tubulin were resuspended in 100 mM sodium *N*-morpholinoethanesulphonate (MES), pH 6.4, 1.0 mM EGTA, 0.5 mM MgCl<sub>2</sub>, and 1.0 mM GTP, and chilled on ice for 1 h for depolymerisation. The suspensions were then centrifuged at 100,000g for 1 h at 4 °C, and the pellets discarded. Aliquots of the supernatants (purified tubulin) were added to mixtures that finally contained: 8.5 µg protein, 33 mM MES, 0.33 mM EGTA, 3.7 mM MgCl<sub>2</sub>, 0.4 mM GTP, and 6.7 mM sodium phosphate, pH 6.8, in a volume of 150 µl; and pre-incubated for various times at 37 °C. At the end of each pre-incubation period, 5 µl of an aqueous 0.186 mM <sup>3</sup>H-colchicine (New England Nuclear) solution (2.16 Ci mmol<sup>-1</sup>) were added to the samples to be tested for binding activity, and an additional incubation was carried out for 1 h at 37 °C. Subsequently the samples were analysed using DEAE-Sephadex column chromatography<sup>11</sup>. All assays were run in triplicate and the data points shown represent the average values. The curve was constructed by linear least-squares fitted to these values. The total time includes pre-incubation plus the 1 h of incubation with <sup>3</sup>H-colchicine. Protein was determined according to the method of Lowry *et al.*<sup>15</sup>.

bulk (about 90%) of the polymers formed consisted of tubules; the rest were ribbons.

A plot of the assembly data of one experiment is shown in Fig. 2, in which the decay was measured over a period of 2 h. As with the decay of colchicine-binding activity, the loss of polymerisability followed apparent first-order kinetics. Its rate seemed to be faster, however, than that of the former by an order of magnitude. The half life was calculated to be only 25.0 ± 1.8 min. Addition of supplementary fresh GTP to the solutions did not affect the amount of polymer formed at any time. Since these data suggested that the decay of some factor other than tubulin itself might have been responsible for the rapid decay of polymerisability, we attempted to supplement C<sub>6</sub> tubulin preparations with microtubule-associated protein,  $\tau$  factor<sup>3</sup>, which has been shown to be essential for the formation of microtubules from preparations of hog brain tubulin. The addition of such hog brain factor to polymerisation mixtures of C<sub>6</sub> tubulin not only increased the amount of C<sub>6</sub> polymers formed in general, but, most interestingly, it induced the formation of tubules even at times after the polymerisability of non-supplemented tubulin had decreased to zero. Table 1 presents data obtained from such an experiment. Note that even after a pre-incubation of 510 min (about 3 h after the polymerisation of the tubulin preparation alone became undetectably low) the amount of polymer formed in the presence of heterologous  $\tau$  factor exceeded by 19-fold the quantity formed with comparatively fresh preparations (60 min pre-incubation) in the absence of added factor.

Our results strongly suggest that the polymerisability of C<sub>6</sub> tubulin preparations depends on the integrity of some



**Fig. 2** Decay rate of polymerisability.  $t_{1/2} = 25.0 \pm 1.8$  min. Preparations of depolymerised, purified tubulin were obtained as described in Fig. 1, except that the pellets of polymerised tubulin were resuspended in 25 mM MES, pH 6.4, 1.0 mM EGTA, 0.1 mM EDTA, 0.5 mM  $MgCl_2$  and 1.0 mM GTP. At the time indicated, 50- $\mu$ l aliquots were incubated for 15 min at 37 °C. A standard solution of tomato bushy stunt virus (BSV) was then added and the mixture sprayed with 5% uranyl acetate, using a dual nebuliser, on to a carbon-coated collodion grid<sup>12</sup>. The number of BSV was counted and the lengths of the microtubules and ribbons measured in three drop patterns for each data point. The percentage of the protein present as polymer was then calculated from these data, with knowledge of the final concentrations of tubulin (6.80 mg ml<sup>-1</sup>) and BSV (0.495 mg ml<sup>-1</sup>)<sup>12</sup>.

endogenous factor other than tubulin itself. Alternatively, it is possible that tubulin could exist in multiple states with the polymerising capacity of a tubulin molecule decaying independently from the colchicine-binding ability and that  $\tau$  factor could reverse the loss of polymerising function. But the simpler explanation, involving only loss of a factor, seems more likely. The decay of this factor must be more rapid than that of the tubulin, since the tubulin could still be polymerised at a time when the activity of this factor was already too low to induce any polymer formation. The data also demonstrate that even initially the concentration of this factor in active form is a limiting element in microtubule assembly from C<sub>6</sub> tubulin.

The experimental conditions used for measuring the decay of colchicine-binding activity and that of polymerisability differed from each other in several aspects, such as the protein concentrations of the assay mixtures, the temperatures of pre-incubations before the tests and the slightly different compositions of the buffer solutions. We believe, however, that the results shown in this report provide a conservative estimate of the divergence between the polymerisability and colchicine-binding decays. For

example, if we had been able to increase the protein concentration of the colchicine-binding assay mixture to the level used for measuring polymerisation, the difference between the two measured decay rates would have been even greater than it was, since the decay of colchicine-binding activity is known to slow down as tubulin concentrations are elevated<sup>1</sup>. Similarly, the performance of pre-incubations at 0–4 °C (before colchicine-binding activity assays) instead of at 37 °C, only lengthened the half-life of the colchicine-binding activity (ref. 4 and G.W., unpublished). The modest levels of glycerol remaining in the tubulin preparations actually resulted in underestimation of the difference in decay rates; the concentration of residual glycerol was 100 times less in colchicine binding than during polymerisation.

In conclusion, we have shown that with tubulin purified from cultured C<sub>6</sub> glial cells, the capacity for microtubule assembly decays at a much faster rate than the tubulin molecules themselves. Presumably, the decay rate of polymerisability expresses primarily alteration or loss of endogenous protein factors that co-purify with tubulin through repeated cycles of *in vitro* polymerisation and depolymerisation.

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**Table 1** Influence of heterologous  $\tau$  factor on polymer formation

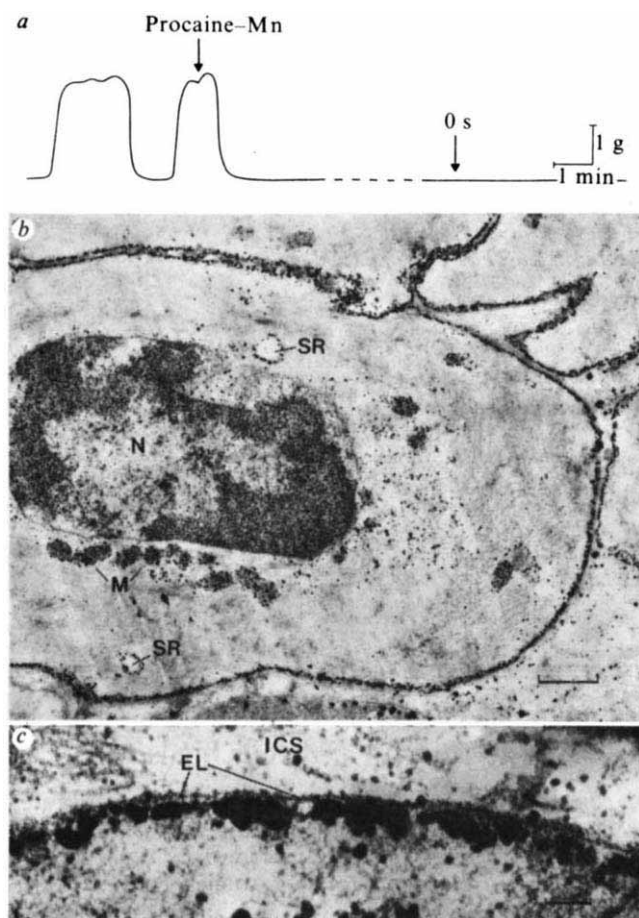
| Pre-incubation time on ice (min) | $\tau$ concentration (mg ml <sup>-1</sup> ) | Percentage protein as polymer |
|----------------------------------|---------------------------------------------|-------------------------------|
| 60                               | 0                                           | 0.28                          |
| 330                              | 0                                           | <0.01                         |
| 510                              | 0                                           | <0.01                         |
| 510                              | 0.61                                        | 5.32                          |

A fresh preparation (see Fig. 2) of depolymerised tubulin (7.5 mg ml<sup>-1</sup>) was incubated at 0–4 °C. At the times indicated, aliquots were taken and incubated for 15 min at 37 °C. The extent of polymer formation (percentage total protein as polymer) was then determined by quantitative electron microscopy as described in Fig. 2. The formation of polymers in the presence of  $\tau$  factor was determined after mixing tubulin and  $\tau$  factor at a ratio of 2:1 (v/v) before incubation at 37 °C. The  $\tau$  factor fraction was isolated by phosphocellulose chromatography<sup>5</sup> from hog brain microtubule protein that had been purified by three cycles of polymerisation and depolymerisation<sup>10</sup>. After elution from the column, the preparation was dialysed against 50 mM ammonium acetate, pH 6.4, and 10 mM 2-mercaptoethanol, concentrated by vacuum dialysis against the same buffer, and then stored frozen. Each data point shown represents the average measurement of three drop patterns.

## Translocation of intracellularly stored calcium during the contraction-relaxation cycle in guinea pig taenia coli

ALTHOUGH it has been shown that, in vertebrate smooth muscles, cation binding sites are localised in some intracellular structures<sup>1–7</sup>, and that isolated microsomal fraction can accumulate Ca in the presence of ATP (refs 8–10), the role of the Ca-accumulating structures in the contraction-relaxation cycle is not firmly established. Using potassium pyroantimonate, which is known to penetrate intact cell membrane in the presence of osmium to produce an electron-opaque precipitate with intracellular cations<sup>11,12</sup>,





**Fig. 1** *a*, Record of isometric tension during the procedure used to determine Ca localisation in the resting fibres. Spontaneous rhythmic contractions were eliminated by the addition of 1 mM procaine and 5 mM Mn ions before the application of the pyroantimonate-osmium solution (Os). *b*, Cross section of the resting fibres showing the localisation of the precipitate around the peripheral part of the fibre. N, nucleus. SR, sarcoplasmic reticulum. Unstained, scale bar 1  $\mu$ m. *c*, High-magnification view around the plasma membrane. The precipitate is mostly located inside the external lamina (EL) of the plasma membrane. ICS, intercellular space. Unstained, scale bar 0.1  $\mu$ m.

experiments on a molluscan smooth muscle have provided evidence that Ca localised in some intracellular structures is released into the myoplasm during mechanical activity<sup>13,14</sup>. We have used this method to investigate the role of the intracellular Ca-accumulating structures in the contraction-relaxation cycle of guinea pig taenia coli. When guinea pig taenia coli was fixed at rest the electron-opaque precipitate containing Ca was observed at or near the plasma membrane. If the fixation was made at the peak of mechanical activity, the precipitate was diffusely distributed in the myoplasm.

Pieces of taenia coli (5–10 mm long) were mounted horizontally in an experimental chamber (3 ml) filled with oxygenated Krebs solution at 37 °C; one end of the preparation was clamped and the other end connected to a strain gauge to record isometric tension. After each experiment the preparation was fixed by replacing the experimental solution with 1% OsO<sub>4</sub> solution (adjusted to pH 7.4 with 0.01 M acetic acid) containing 2.5% potassium pyroantimonate (K[Sb(OH)<sub>6</sub>]), dehydrated with ethanol, and embedded in Epon 812. Sections with gold colour were examined with a Hitachi HU-12 electron microscope.

The localisation of Ca-accumulating structures in the resting muscle fibres was examined in the following way.

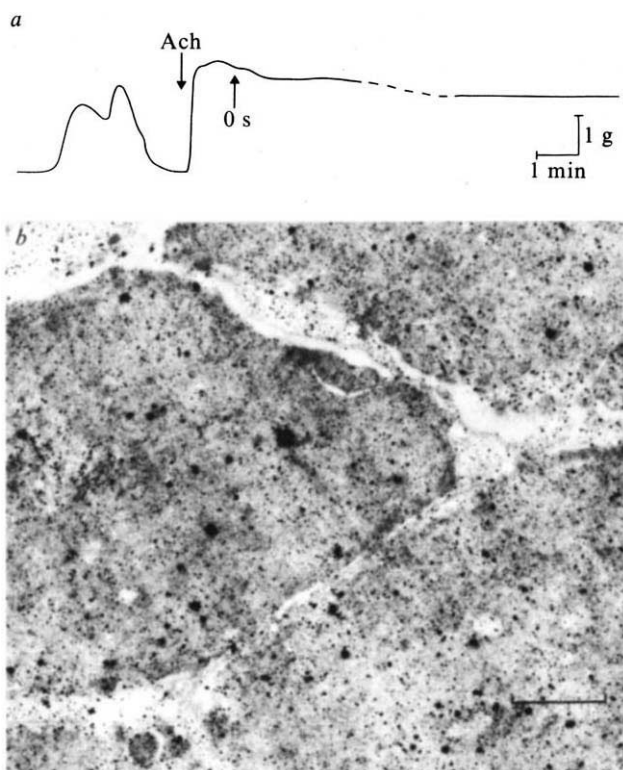
The preparation was first kept in Krebs solution for more than 20 min, and immersed in isotonic KCl solution containing 2.5 mM potassium pyroantimonate for 10 min to introduce the pyroantimonate into the fibres during membrane depolarisation (it did not readily penetrate polarised resting membrane). The preparation was then returned to Krebs solution, and after 5–10 min procaine (2 mM) and Mn ions (5 mM) were added to eliminate spontaneous mechanical activity (Fig. 1*a*); the preparation was fixed with the pyroantimonate-osmium solution with no detectable tension development.

Figure 1*b* shows the cross section of the resting fibres fixed by the above method. It can be seen that particles of electron-opaque precipitate are mostly located around the peripheral part of the fibre. Examination of the plasma membrane with higher magnifications (Fig. 1*c*) indicates that a large amount of precipitate is located inside the external lamina of the plasma membrane (probably at the inner surface of the plasma membrane and at the sarcoplasmic reticulum). The precipitate was also observed at the nucleus. Similar localisation of calcium oxalate deposits has been reported in guinea pig taenia coli fixed after treatment with potassium oxalate<sup>4</sup>.

In other experiments the preparation was made to contract with acetylcholine (10<sup>-5</sup> M) and fixed at the peak of resulting contracture tension (Fig. 2*a*). The fall of tension at the completion of fixation was 20–30%. As shown in Fig. 2*b*, the precipitate was diffusely distributed in the myoplasm in the form of a number of particles, while little precipitate was seen at the periphery of the fibre. The amount of precipitate at the nucleus apparently remained unchanged. Similar results were obtained in the fibres fixed at the peak of spontaneous contractions.

For chemical identification of the precipitate, the sections

**Fig. 2** *a*, Record of contracture tension induced by 10<sup>-5</sup> M acetylcholine (ACh), and its decline after 30 min in the pyroantimonate-osmium solution (Os). *b*, Cross section of the fibres fixed at the peak of the contracture tension. The precipitate is diffusely distributed in the myoplasm in the form of a number of particles, while it is hardly observable around the peripheral part of the fibre. Unstained, scale bar 1  $\mu$ m.



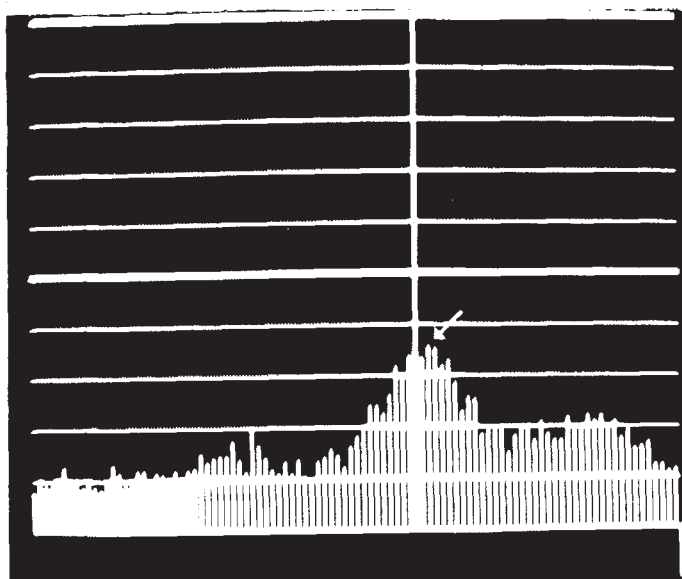


Fig. 3 X-ray spectrograph of the precipitate. The peak at 3,620 eV (arrow) is regarded as a combined spectrum of Sb-L $\alpha$  and Ca-K $\alpha$  lines. Thick vertical line indicates the standard peak of Sb-L $\alpha$  line (3,600 eV).

were analysed with an energy dispersive X-ray microanalyser (EDAX-707A) attached to a Hitachi HHS-2R electron microscope (accelerating voltage 25 kV, counting time 400 s). The X-ray spectrograph of the precipitate in both the resting and the contracted fibres showed a distinct peak at 3,620 eV (Fig. 3). Model tests with Ca and antimonate<sup>15</sup> suggest that this spectrum results from the combination of Sb L-line (3,600 eV) with Ca K-line (3,690 eV). This indicated the presence of calcium pyroantimonate in the precipitate. Quantitative analysis of the precipitate was further made with another energy dispersive X-ray microanalyser (EDAX-707B) attached to a JEOL JEM-100C electron microscope together with a computerised EDIT system (accelerating voltage 40 kV, counting time 400 s). As shown in Table 1, the result again showed the presence of Ca in the precipitate in both the resting and the contracted fibres, though its concentration ratio was smaller than those of K and Na. The presence of Mg was not detected. But, considering that the amount of Ca ions involved in the activation of the contractile system is far smaller than those of K and Na ions in the myoplasm<sup>16</sup>, the values of concentration ratio of Ca in the precipitate may be taken to indicate that the precipitate serves as a measure of Ca localisation.

In conclusion, the translocation of the precipitate containing Ca from the peripheral intracellular structures into the myoplasm during mechanical activity seems to be consistent with the view that, in guinea pig taenia coli, the contraction-relaxation cycle is regulated by the release of Ca from, and its uptake by, the intracellular Ca-accumulating structures.

We thank Dr Suechika Suzuki and Miss Yoko Narikawa

Table 1 Quantitative analysis of elemental concentration ratios in the precipitate

| Element | Line       | Mean ratios     |                   |
|---------|------------|-----------------|-------------------|
|         |            | Resting fibres  | Contracted fibres |
| Sb      | L $\alpha$ | 1.00            | 1.00              |
| Ca      | K $\alpha$ | 0.19 $\pm$ 0.07 | 0.12 $\pm$ 0.05   |
| K       | K $\alpha$ | 0.36 $\pm$ 0.12 | 0.57 $\pm$ 0.06   |
| Na      | K $\alpha$ | 0.23 $\pm$ 0.10 | 0.35 $\pm$ 0.12   |

Values are means  $\pm$  s.e.m. Data for resting fibres from seven specimens, for contracted fibres from five specimens.

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## Tumour-specific transplantation antigen from SV40 transformed cells binds to DNA

MUCH work is being directed towards the characterisation of tumour-specific transplantation antigens (TSTA). AL/N strain mouse embryo cells transformed by SV40 virus (SV AL/N cells) are suitable for such studies, because (1) these cells are highly antigenic and are rejected at high dose ( $>10^7$ ) by the immunologically competent syngeneic AL/N mouse<sup>1,2</sup>, (2) TSTA is common to all SV40 virus-transformed cells tested but specific in that only SV40 transformed cells share a common TSTA<sup>3</sup>, and (3), the SV40 'early' or A gene is relatively small ( $\sim 1.8 \times 10^6$  molecular weight), its mapping and the nucleotide sequences of the restriction endonuclease fragments are under intense study, and the various cellular antigens in the transformed cells—the TSTA, the tumour-specific surface antigen (TSSA) (ref. 4), and the nuclear T antigen—probably represent modifications of products of this early gene<sup>5-7</sup>. Using SV AL/N cells and a detergent technique<sup>8</sup> for solubilisation, we demonstrated that immunisation by a partially purified soluble extract containing 100  $\mu$ g protein effectively protects mice against tumourigenic doses of SV40 transformed cells<sup>9</sup>. We report here that TSTA activity co-purifies with the T antigen in several steps, that TSTA can be recovered from a DNA column in a fraction which then effectively immunises at the 1- $\mu$ g level, and that the TSTA, like the T antigen<sup>10</sup>, binds to double-stranded mammalian DNA.

Nuclear preparations from an SV40 transformed human cell line are rich in TSTA activity<sup>5</sup>. Accordingly, fractions from murine SV AL/N cells enriched in nuclei and also containing the cytoplasmic membranes were sonicated and solubilised by the detergent technique. Details are given in Table 1. Antigen enrichment was by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  followed by DEAE-cellulose chromatography<sup>11</sup>. A large amount of the T-antigen activity of the whole extract was recovered in the  $(\text{NH}_4)_2\text{SO}_4$  precipitate (84%), and in a fraction which eluted at about 0.15 M NaCl from the DEAE column (40% of input; Table 1 and Fig. 1). This pooled fraction was enriched in TSTA activity: it gave significant ( $\sim 75\%$ ) and specific protection at the 4- $\mu$ g level, and full protection at the 20- $\mu$ g level (Table 2).

The pooled DEAE fractions were then chromatographed on a DNA-cellulose column<sup>10</sup>. About 70% of the T-antigen activity was recovered in a sharp peak (pool III, Fig. 2),



**Table 1** Distribution and recovery of T-antigen activity

|                                                |          | CF units<br>( $\times 10^3$ ) | Protein<br>(mg) | Specific activity<br>(CF units $\text{mg}^{-1}$ )<br>$\times 10^2$ |
|------------------------------------------------|----------|-------------------------------|-----------------|--------------------------------------------------------------------|
| Whole extract                                  |          | 310                           | 82              | 37.5                                                               |
| 0–50% $(\text{NH}_4)_2\text{SO}_4$ precipitate |          | 255                           | 60              | 42                                                                 |
| DEAE column                                    | input    | 235                           |                 |                                                                    |
| pool                                           | recovery | 95                            | 12              | 50                                                                 |
| DNA column                                     | input    | 59                            |                 |                                                                    |
| pool III                                       | recovery | 42                            | 3               | 140                                                                |

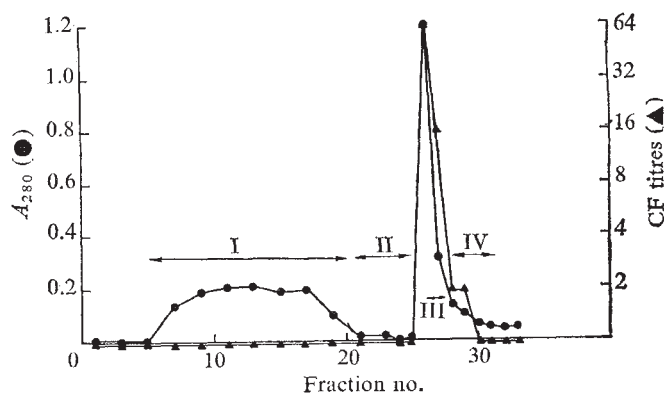
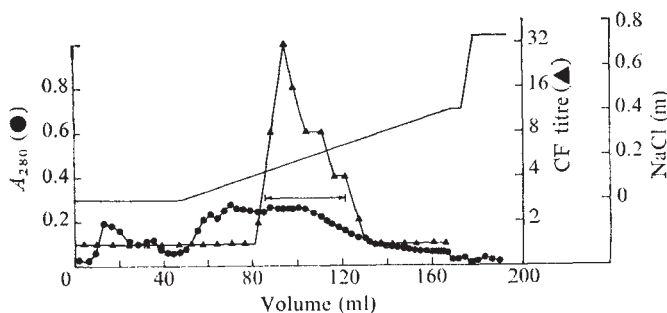
Subconfluent SV40 transformed AL/N murine embryo cells (SV AL/N line) were grown as before<sup>2</sup>, the cells were collected by scraping, and washed three times with Tris-buffered saline, pH 7.4. To the washed cells was added three times the pellet volume of ice-cold buffer containing 10 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1.5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol (DTT), 100  $\mu\text{M}$  L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) and 100  $\mu\text{M}$  phenylmethylsulphonylfluoride (PMSF) (buffer I). The suspension was homogenised by four strokes on a Dounce homogeniser, and centrifuged at 700g for 10 min. The pellet, containing mainly nuclei and cytoplasmic membranes, was suspended in three times its volume of buffer I, given four strokes on a Dounce homogeniser and centrifuged again at 700g for 10 min. The pellet was resuspended in buffer II containing 0.02M Tris-HCl, pH 8.2, 0.08M NaCl, 1mM DTT, 10% glycerol, 0.001% Triton X-100, 100  $\mu\text{M}$  each TPCK and PMSF<sup>11</sup>. The suspension was sonicated for 1 min and centrifuged at 27,000g for 1 h. The pellet was resuspended in the same volume of buffer II, homogenised and centrifuged as before. The two supernatants were combined, designated as whole extract, and precipitated with ammonium sulphate to a final concentration of 50%. Further fractionation was achieved on a DEAE-cellulose column as in Fig. 1, followed by a DNA-cellulose column as in Fig. 2. T antigen was measured by standard microtitre complement fixation assay, and units were calculated as in ref. 6. Anti-T antiserum was provided by Dr J. Gruber (NCI). Protein was determined by the Lowry technique<sup>12</sup>. Values are in complement-fixing (CF) units.

with about threefold increase in specific activity (Table 1).

When the pooled fractions from the DNA-cellulose column were tested for TSTA, only those fractions which bound to calf thymus DNA had this activity. More than 50% protection was provided against 10–100-fold tumourigenic doses of mKSA-ASC tumour cell challenge by 1  $\mu\text{g}$ , and full protection of 5  $\mu\text{g}$ , of pool III material. Pooled fractions I and II, which did not bind to DNA and had no T antigen activity, were found to be inactive in the TSTA test at 40–60- $\mu\text{g}$  level. The pooled fraction IV, which bound to the DNA column, had significant TSTA activity at the 10- $\mu\text{g}$  level (Table 2).

It is significant that the T antigen and TSTA from SV40 transformed AL/N cells co-purify through ammonium

**Fig. 1** Elution of T antigen from DEAE-cellulose column. The 0–50% ammonium sulphate precipitate (see legend to Table 1) was dialysed against buffer III containing 0.05M Tris-HCl pH 7.8, 1 mM DTT, 100  $\mu\text{M}$  each TPCK and PMSF, and 10% glycerol. The dialysed material was chromatographed on a DEAE-cellulose (DE-52, Whatman) column (8  $\times$  2 cm), eluted by a gradient of 0–0.4M NaCl in buffer III, as reported previously<sup>11</sup>. The CF titre recovered is the reciprocal of the dilution of a 25- $\mu\text{l}$  aliquot which fixes 50% of the complement<sup>6</sup>. The peak of T-antigen activity eluted at 0.15 M NaCl, as observed before<sup>11,16</sup>. The fractions designated by the horizontal bar were pooled, and used for the DNA column fractionation (Fig. 2).



**Fig. 2** Elution of T antigen from DNA-cellulose column. The pooled fractions from the DEAE-cellulose column (Fig. 1), were dialysed against 5 mM potassium phosphate buffer, pH 6.2, containing 0.1 M NaCl, 1mM DTT, 100  $\mu\text{M}$  each TPCK and PMSF, and 10% glycerol. A double-stranded DNA-cellulose column (12  $\times$  1 cm) was made from Sigma calf thymus DNA as before<sup>10</sup>. The potassium phosphate buffer was replaced, when fraction number 22 eluted, with buffer IV containing 10 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM DTT, 100  $\mu\text{M}$  each TPCK and PMSF, and 10% glycerol, to elute T-antigen activity<sup>10</sup>. Fractions were analysed for ▲, T antigen (CF) and ●, absorbance (280 nm), and pooled as defined by the horizontal bars, for TSTA determination (see Table 2).

sulphate precipitation, DEAE- and DNA-cellulose column chromatography, and that both T and TSTA specific activities were enriched through the binding to the DNA.

By studying temperature sensitive SV40 mutant viruses defective in the early A region (the tsA mutants)<sup>12</sup>, it was found that the expression of T and TSTA is modulated coordinately<sup>6,13,14</sup>. Adeno-SV40 hybrid viruses with SV40 T and/or TSTA antigen activity induce proteins with apparently overlapping regions in their polypeptide

**Table 2** TSTA activity

| Immunisation with | Dosage ( $\mu\text{g}$ protein) | Tumour cells for challenge | Tumours/total inoculated | P values |
|-------------------|---------------------------------|----------------------------|--------------------------|----------|
| Buffer I          |                                 | $10^4$ mKSA-ASC            | 10/10                    |          |
|                   |                                 | $10^4$ Meth-1-A            | 6/10                     |          |
| Whole extract     | 4                               | $10^4$ mKSA-ASC            | 5/7                      |          |
|                   | 20                              | $10^4$ mKSA-ASC            | 3/7                      | < 0.015  |
|                   | 100                             | $10^4$ mKSA-ASC            | 1/7                      | < 0.003  |
|                   | 20                              | $10^4$ Meth-1-A            | 5/10                     |          |
| DEAE pool         | 1                               | $10^4$ mKSA-ASC            | 7/8                      |          |
|                   | 4                               | $10^4$ mKSA-ASC            | 2/8                      | < 0.002  |
|                   | 20                              | $10^4$ mKSA-ASC            | 0/8                      | < 0.002  |
| Buffer IV         |                                 |                            | 15/15                    |          |
| DNA pool I        | 3                               | $10^4$ mKSA-ASC            | 7/7                      |          |
|                   | 15                              | $10^4$ mKSA-ASC            | 8/8                      |          |
|                   | 60                              | $10^4$ mKSA-ASC            | 8/8                      |          |
| DNA pool II       | 10                              | $10^4$ mKSA-ASC            | 8/8                      |          |
|                   | 40                              | $10^4$ mKSA-ASC            | 7/8                      |          |
| DNA pool III      | 1                               | $10^4$ mKSA-ASC            | 3/7                      | < 0.005  |
|                   | 5                               | $10^4$ mKSA-ASC            | 0/8                      | < 0.002  |
|                   | 25                              | $10^4$ mKSA-ASC            | 0/8                      | < 0.002  |
| DNA pool IV       | 0.2                             | $10^4$ mKSA-ASC            | 8/8                      |          |
|                   | 2                               | $10^4$ mKSA-ASC            | 7/8                      |          |
|                   | 10                              | $10^4$ mKSA-ASC            | 2/8                      | < 0.002  |

Adult BALB/c mice were injected intraperitoneally (i.p.) twice with the indicated dose of protein at a 1-week interval. Ten days after the second injection the mice were challenged i.p. with the indicated number of the SV40 transformed BALB/c mKSA-ASC cells<sup>17,18</sup> (provided by Dr L. W. Law), or intramuscularly with the methylcholanthrene transformed Meth-1-A BALB/c cells which carry their own TSTA<sup>19</sup>, for specificity control<sup>20</sup>. The  $\text{TD}_{50}$  of the mKSA-ASC cell<sup>18</sup> is  $10^2$ – $10^3$ , the  $\text{TD}_{50}$  of the Meth-1-A is about  $10^4$  cells. Evidence of ascites tumour appeared by about 2 weeks in control mice, with a mean time to death of 20 d after challenge. Inoculated mice were followed for 5 weeks. The differences in the P values<sup>21</sup> between immunised and non-immunised groups are significant where given.



sequences<sup>7</sup>. Nuclear fractions from SV40 transformed human cells are enriched in T antigen and also in TSTA<sup>8</sup>.

Our findings give independent confirmation of the suggested homology between T antigen and TSTA, two of the biologically important products of the early SV40 gene in SV40 transformation. Furthermore, the binding of TSTA to double-stranded mammalian DNA opens the possibility of molecular study of the paradoxical dual role of this protein: first as a tumour rejection antigen *in vivo*, and second as a candidate regulatory protein for the growth of cells in tissue culture.

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## Productive T7 infection of *Escherichia coli* F<sup>+</sup> cells and anucleate minicells

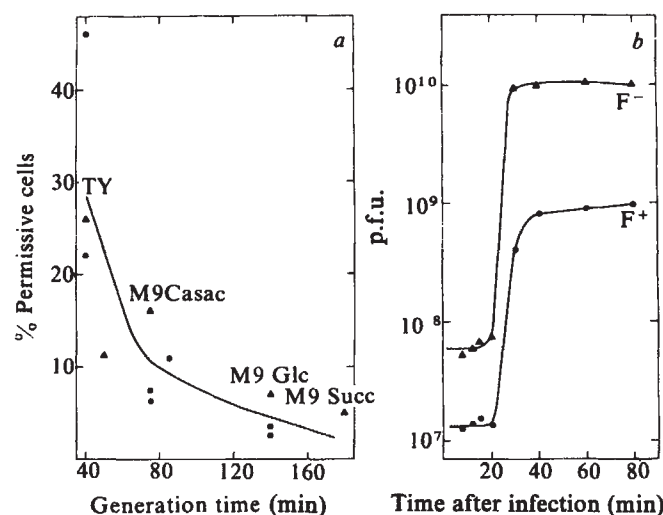
THE development of colivirus T7 is largely independent of host cell functions and dependent on T7-encoded enzymes, for example, T7 RNA polymerase, DNA replication enzymes, and lysozyme<sup>1,2</sup>. In view of this relative independence, the failure to propagate in *Escherichia coli* cells carrying an F factor<sup>3,4</sup> or in anucleate minicells<sup>5</sup> is particularly interesting with respect to a molecular understanding of host-virus interaction. We report here that T7 can, at a low frequency, productively infect both F<sup>+</sup> cells and minicells derived from either an F<sup>+</sup> or an F<sup>-</sup> *E. coli* culture. The percentage of T7 permissive cells within an F<sup>+</sup> population is dependent on the growth conditions of the culture but does not seem to be directly related to a specific phase of the cell cycle.

T7 produces approximately 10,000 times more plaques when plated on an F<sup>-</sup> lawn of *E. coli* cells compared with F<sup>+</sup> lawns. The plaques produced on an F<sup>+</sup> lawn are irregular in size and do not contain selected mutant or host-cell-modified phage, as phages isolated from these plaques have no increased ability to grow on lawns of F<sup>+</sup> cells. All attempts to isolate mutants of T7 capable of plating on F<sup>+</sup> and F<sup>-</sup> cells at the same efficiency, have so far failed. Although previous results suggest a role for a T7 early product in the exclusion of T7 by F<sup>+</sup> cells<sup>6</sup>, we have observed exclusion of T7 deletion mutants lacking all early functions. Thus, it seems likely that the 'non-permissiveness' cannot be overcome by mutations in T7. This led us to examine factors related to the F<sup>+</sup> *E. coli* cell which determine the 'non-permissiveness'.

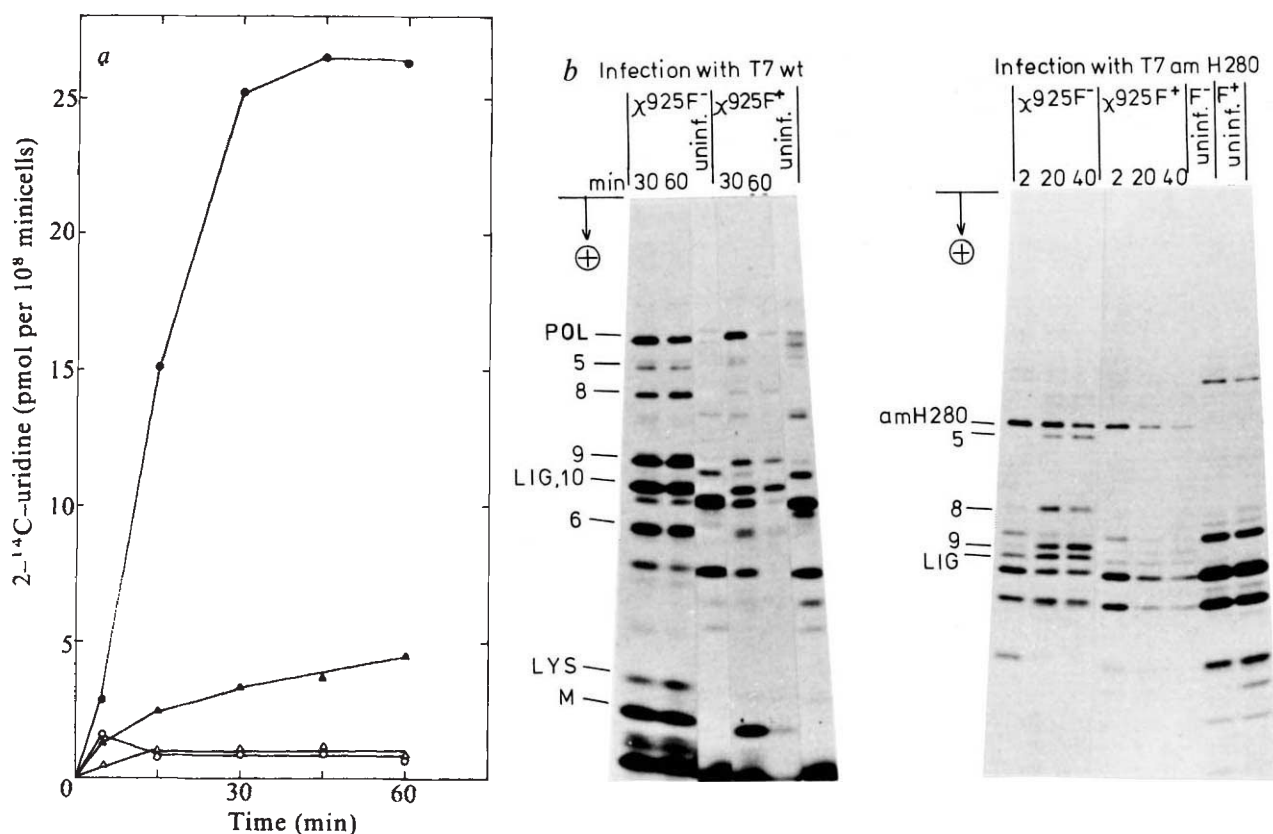
After quantitation, we found that 2-50% of an F<sup>+</sup> cell population could propagate T7, depending on growth conditions (Fig. 1a). F<sup>+</sup> cells were grown in various media, infected with T7, and the infected cells plated with F<sup>-</sup> indicator bacteria. A culture grown in rich medium contained the highest number of T7 permissive cells. Culture conditions therefore influence strongly the fate of T7 in F<sup>+</sup> cells, by altering the ratio of permissive and non-permissive cells. The permissive F<sup>+</sup> cells release a burst of T7 comparable in size with that released from F<sup>-</sup> cells (Fig. 1b), indicating that once an F<sup>+</sup> cell is committed to permissiveness the subsequent T7 development is normal.

In the same F<sup>+</sup> cell culture, there seem to be two types of cells. This heterogeneity could not result from segregation of the F factor as identical results were obtained when growth was dependent on the presence of the F factor. As explanations for the heterogeneity we will consider the following possibilities: (1) productive infection occurs in cells which lose the plasmid during infection, for example, infection of cells in the process of cell division; (2) the permissive cells retain the plasmid but pass through a T7 permissive phase during the cell cycle; (3) each cell at any stage of the cell cycle can be infected either productively or abortively, depending on a statistical event during the infection process.

The observation that the growth rate of a culture of F<sup>+</sup> cells altered the percentage of T7 permissive cells, suggested that the heterogeneity within the population for T7 permissiveness may reflect a stage within the *E. coli* life cycle. We have attempted to demonstrate an increase in the number of productively infected cells at some stage in the cell cycle by infecting *E. coli* F<sup>+</sup> cells synchronised by a variety of techniques (that is, thymine and amino acid starvation,



**Fig. 1** Detection of T7 permissive F<sup>+</sup> cells. *a*, *E. coli* 803 F<sup>-</sup> and 803 F<sup>+</sup> (ref. 6) were grown in various media and, at a cell density of  $5 \times 10^8$  ml<sup>-1</sup>, infected with T7 am 193<sup>+</sup>K<sup>+</sup> (ref. 6) at a multiplicity of infection of 10 (30 °C). After 5 min, T7 antiserum was added and allowed to act for 3 min. Then the p.f.u.s were determined by plating aliquots with *E. coli* 803 F<sup>-</sup> indicator bacteria. The p.f.u.s indicate T7 permissive cells. The p.f.u.s obtained with 803 F<sup>+</sup> were plotted as a percentage of the total cell count against generation time of the bacteria. Total cell count was equal to the number of p.f.u. obtained in the F<sup>-</sup> culture. Generation time varied with the culture conditions: tryptone-yeast broth, 40 min; M9 0.4% glucose supplemented with casamino acids, thymine, and thiamine<sup>11</sup>, 50 min; M9 0.4% glucose supplemented with 0.03% casamino acids, 75-85 min; M9 0.4% glucose, 130 min and M9 0.4% succinate, 170 min. *b*, One-step growth curves of T7 development in *E. coli* F<sup>-</sup> and F<sup>+</sup>. As in *a*, 803 F<sup>-</sup> and F<sup>+</sup> were grown in tryptone-yeast medium and infected, at a density of  $5 \times 10^8$  ml<sup>-1</sup>, with T7 am 193<sup>+</sup>K<sup>+</sup>. After 5 min, T7 antiserum was added and, at 8 min, the culture was diluted 1:10<sup>4</sup> to dilute out the antibodies. p.f.u.s were determined at various times thereafter, by plating on 803 F<sup>-</sup> bacteria.



**Fig. 2** T7 gene expression in anucleate minicells derived from F<sup>-</sup> and F<sup>+</sup> strains. The minicell producing strain χ925 (ref. 15) and an isogenic F<sup>+</sup> derivative were grown in L broth to  $A_{600} = 0.8$ . Minicells were separated from nucleated parental cells as described previously<sup>16,17</sup>. **a**, <sup>14</sup>C-uridine incorporation. Minicells ( $2 \times 10^9$  ml<sup>-1</sup>) were infected with T7 at 0 °C in M9 glucose minimal medium containing 10 μg ml<sup>-1</sup> threonine and 10 μg ml<sup>-1</sup> leucine (multiplicity of infection = 20). 2-<sup>14</sup>C-uridine (0.11 μCi ml<sup>-1</sup>, 59 mCi mmol<sup>-1</sup>) was added and the suspensions were placed at 30 °C. Samples (100 μl) were taken at various times, mixed with 100 μl lysozyme solution (2 mg lysozyme per ml TES, that is, 0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.05 M EDTA) and rapidly frozen by immersion of the tubes in liquid nitrogen. When all samples had been taken they were simultaneously placed at 37 °C for 2 min; 20 μl of 2% sarkosyl in TES was added followed by 2 ml ice-cold 5% TCA. Cold TCA-precipitable material in each sample was measured as described previously<sup>18</sup>. ●, F<sup>-</sup> minicells, infected; ○, uninfected; ▲, F<sup>+</sup> minicells, infected; △, uninfected. **b**, Protein synthesis. Minicells were resuspended in M9 glucose minimal medium containing 10 μg ml<sup>-1</sup> threonine and 10 μg ml<sup>-1</sup> leucine and were infected with either T7 am 193<sup>+</sup>K<sup>+</sup> or T7 am H280 (T7 RNA polymerase<sup>-</sup>, protein kinase<sup>-</sup>) at a multiplicity of infection of 10. At various times (as indicated), <sup>14</sup>C-amino acids were added (25 μCi ml<sup>-1</sup>). After a further 20 min of incubation, the infected minicells were removed from the radioactive medium by centrifugation, and dissolved in sample buffer for polyacrylamide gel electrophoresis. Autoradiograms of 10–20% gradient gels are shown. This figure, in addition to the information discussed in the text, covers two aspects. (1) The expression of stable mRNA in minicells<sup>18</sup> can be detected. After infection with T7, the expression of this mRNA is stopped, indicating translational control<sup>19</sup>. (2) A comparison between products made from stable mRNA in the minicells derived from F<sup>-</sup> and F<sup>+</sup> cells, reveals one major difference. One additional peptide of molecular weight of about 25,000 is synthesised in the F<sup>+</sup> minicells. Thus, the sex factor also seems to code for an extremely stable messenger.

use of *dna*<sup>-</sup>ts mutants, successive fractionation of progeny cells, and fractionation of cells according to size). We have so far been unable to demonstrate any relationship between the division cycle of *E. coli* and productive T7 infection. The possibility still exists that the techniques used for synchronising cell division, did not synchronise the event responsible for T7 permissiveness (for example, the state of F factor replication). Although negative, these results argue against our first two suggestions for T7 permissiveness of F<sup>+</sup> cells.

Inhomogeneity in each cell may be mediated by the cell envelope. Inhomogeneities of the cellular surface with respect to newly synthesised areas have, for example, been found using phage receptors as probes<sup>7,8</sup>. The role of the membrane in the abortive infection of F<sup>+</sup> cells by T7 has indeed been documented<sup>9,10,11</sup>, and we know from earlier experiments that no soluble factor can be responsible for the exclusion<sup>6</sup>. We hypothesise, therefore, that the membrane carries 'permissive' and 'non-permissive' entry sites for T7. Depending on the growth conditions, the episome influences the ratio of the two sites. From this hypothesis we expect that after loss of the episomal DNA, cells would regain up to 100% T7 permissiveness, dependent on growth of the cell envelope, but would retain the ability to exclude

T7 if no growth occurred. These two conditions were tested using *E. coli* carrying the temperature-sensitive *F*<sub>tsH</sub> lac episome and using minicells derived from F<sup>+</sup> parent cells.

After shift of an *E. coli* *F*<sub>tsH</sub> lac culture from 30 °C to 42 °C, F replication stops and the F DNA is diluted out with cell division. The re-appearance of T7 permissiveness followed the segregation kinetics with a brief lag period (not shown). These data would be consistent with the assumption of a continuous regeneration of permissive membrane sites.

Conditions in which the cellular surface is not significantly altered after loss of DNA, are found with anucleate minicells. We therefore constructed isogenic F<sup>+</sup> and F<sup>-</sup> strains of an *E. coli* mutant which produces anucleate minicells. The minicells formed do not contain chromosomal or episomal DNA. After infection of minicells from the F<sup>-</sup> strain, uridine was rapidly polymerised into a TCA-precipitable form (RNA), whereas the infected F<sup>+</sup> minicells synthesised very much less RNA (Fig. 2a). The uninfected F<sup>+</sup> and F<sup>-</sup> minicells did not produce significant amounts of TCA-precipitable material. This difference in the patterns of RNA synthesis is very reminiscent of the difference observed in nucleated cells<sup>6,12</sup>. The initial rate of synthesis is similar between F<sup>-</sup> and F<sup>+</sup> nucleated cells whereas at later times the

Table 1 T7 development and recombination in *E. coli* minicells

| Expt | Minicells (ml <sup>-1</sup> ) | Mating type of parental cells | Plaque-forming units (p.f.u.) ml <sup>-1</sup> resulting from infection of minicells by: |                         |                         |                        | p.f.u. resulting from minicells coincidentally infected by pairs of T7 mutants | p.f.u. resulting from minicells singly infected by T7 mutants and subsequently mixed | Viable cell count (ml <sup>-1</sup> ) |
|------|-------------------------------|-------------------------------|------------------------------------------------------------------------------------------|-------------------------|-------------------------|------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------|
|      |                               |                               | T7 <sup>+</sup>                                                                          | T7 gene 11 <sup>-</sup> | T7 gene 17 <sup>-</sup> | T7 gene 1 <sup>-</sup> |                                                                                |                                                                                      |                                       |
| 1    | 2 × 10 <sup>10</sup>          | F <sup>-</sup>                | 8 × 10 <sup>7</sup>                                                                      | < 10 <sup>3</sup>       | 2 × 10 <sup>4</sup>     | —                      | 3.3 × 10 <sup>6</sup>                                                          | 1.8 × 10 <sup>4</sup>                                                                | 1 × 10 <sup>2</sup>                   |
| 2    | 1 × 10 <sup>9</sup>           | F <sup>-</sup>                | 4 × 10 <sup>6</sup>                                                                      | < 10 <sup>3</sup>       | —                       | < 10 <sup>3</sup>      | 2 × 10 <sup>4</sup>                                                            | —                                                                                    | 1 × 10 <sup>3</sup>                   |
|      | 1 × 10 <sup>9</sup>           | F <sup>+</sup>                | 5 × 10 <sup>5</sup>                                                                      | < 10 <sup>3</sup>       | —                       | < 10 <sup>3</sup>      | 3 × 10 <sup>3</sup>                                                            | —                                                                                    | 1 × 10 <sup>3</sup>                   |

Before infection, minicells were (expt 1) or were not (expt 2) pre-incubated in L broth containing 20 µg cycloserine ml<sup>-1</sup>, at 37 °C for 1 h. The multiplicity of infection was 10 (expt 1) or 5 (expt 2). After 5 min at 37 °C, T7 antiserum was added. At 7 min, the infective centres (p.f.u.) were determined by plating with B<sub>8-1</sub> (B strain, *su*<sup>-</sup>) as indicator bacteria. The following viral mutants were used<sup>20</sup>: T7 am 37 (gene 11<sup>-</sup>); T7 am 145 (gene 17<sup>-</sup>) and T7 am 27 (gene 1<sup>-</sup>). To confirm that recombination had occurred within the minicells, we mixed singly infected minicells after the antiserum treatment and plated for infective centres after additional 5 min of incubation. A 50-fold increase in the parental cell count (by addition) did not change the number of infective centres obtained.

synthesis of RNA in the F<sup>+</sup> cell terminates because of the general shut-down of macromolecular syntheses<sup>6,12</sup>.

An unequivocal comparison of specific viral gene products was performed at the level of protein synthesis. Whereas infected F<sup>-</sup> minicells synthesised all early and late T7 proteins, F<sup>+</sup> minicells produced only the early proteins, again in parallel with the process in nucleated cells (Fig. 2b). Both late genes transcribed by the viral RNA polymerase and late genes transcribed by the host RNA polymerase (as in the polymerase-protein kinase double mutant H280), were affected. At later times, early protein synthesis in F<sup>+</sup> minicells was also decreased as in whole cells. From these experiments we conclude that minicells are efficiently infected with T7 and that they produce T7 products in the same order of magnitude as do nucleated cells. This provided evidence for the existence of sufficient host cell RNA polymerase in the minicells to permit transcription of infecting phage genomes as has been described previously for phage infection of minicells produced by *Bacillus subtilis*<sup>13</sup>. The time course of synthesis is, however, expanded by a factor of 5–10 (Fig. 2b). Thus, in minicells a factor must be rate-limiting which is not limiting in nucleated cells. In addition, infection of minicells with T7 carrying a known amber mutation led to the loss of the affected protein and production of the corresponding amber fragment (Fig. 2b), indicating that recognition of nonsense codons occurs normally in minicells.

To determine whether the F<sup>+</sup> minicells were heterogeneous and contained a fraction of permissive cells, we examined the development of virus particles in F<sup>-</sup> and F<sup>+</sup> minicells. Although only little DNA replication was detectable by thymidine incorporation (not shown), a low but significant and reproducible number of F<sup>-</sup> minicells released virus particles (0.1%). Among the infected F<sup>+</sup> minicells (grown in rich medium), a fraction of about 0.01% propagated T7. This is similar to the situation in nucleated cells in which a fraction of the cells is also T7 permissive (Fig. 1a and b).

We were unable to demonstrate a one step growth curve with minicells and would suggest that this is due to a very asynchronous release of a small number of progeny phage. Because of the inherent problems in demonstrating that only one out of 1,000 or 10,000 infected minicells produced phage, the results were confirmed by recombination experiments (Table 1). A significant number of recombinant virus was detected after doubly infecting F<sup>-</sup> and F<sup>+</sup> minicells with T7 amber mutants.

In conclusion, we believe that the results reported here indicate that T7 development in F<sup>+</sup> cells is abortive or permissive, dependent on the growth conditions of the *E. coli* cell culture. The decision as to successful or abortive infection is made at the time of infection and contained within the existing structure of the cell as minicells which

do not contain genetic information show the same properties as do whole cells.

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## Ratios of $\alpha$ -to $\beta$ -globin mRNA and regulation of globin synthesis in reticulocytes

THE genomes of several mammalian species contain at least two  $\alpha$  globin loci and a single  $\beta$  globin locus so that the ratio of  $\alpha$ -to  $\beta$ -globin genes is 2 : 1 or more<sup>1–3</sup>. Despite this difference in number of genes, reticulocytes from these species synthesise equal numbers of  $\alpha$ - and  $\beta$ -globin chains indicating that compensatory regulation must occur. Cytoplasmic mRNA is an intermediate between the gene and globin protein, and it has been suggested that the cellular  $\alpha/\beta$  mRNA ratio is an important factor in achieving balanced globin synthesis<sup>4–6</sup>. We report here an excess of 30%  $\alpha$  mRNA on reticulocyte polysomes of rabbits, mice, and sheep. Our data also illustrate the potential for errors in studies in which only poly(A)<sup>+</sup> RNA is assayed.

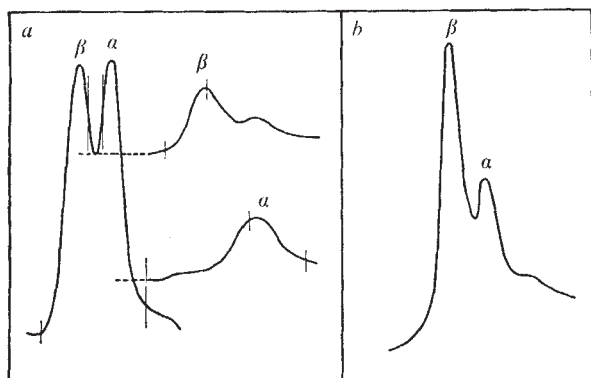
Using inhibitors of polypeptide elongation in rabbit reticulocytes, Lodish has demonstrated that  $\beta$  mRNA initiates polypeptide synthesis 40% faster than  $\alpha$  mRNA; and since the rate of elongation of the two globin polypeptides is equal, he inferred that a 40% excess of  $\alpha$  mRNA on polysomes is required to achieve balanced globin



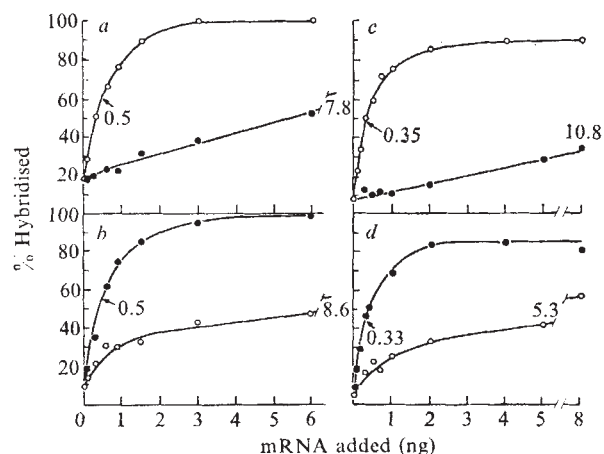
synthesis<sup>5</sup>. Others have inferred a functional  $\alpha/\beta$  RNA ratio of 1.3:1.8 in rabbits<sup>6</sup>.

Attempts to measure directly the molar ratio of  $\alpha$  to  $\beta$  mRNA in reticulocytes have led to conflicting results. Formamide gel electrophoresis of poly(A)<sup>+</sup> RNA obtained by oligo(dT)-cellulose chromatography has yielded  $\alpha$  to  $\beta$  mRNA ratios as low as 1 and as high as 1.6 (refs 7-12). Recently single determinations using hybridisation with cDNA probes of 85% specificity have given  $\alpha/\beta$  RNA ratios of 1.1 and 1.5 in total RNA of mouse and poly(A)<sup>+</sup> RNA of human reticulocytes<sup>12,15</sup>.

Globin mRNAs of rabbit, mouse and sheep were separated by formamide gel electrophoresis<sup>7-12</sup>. Although these mRNA templates seemed 80-90% pure by gel analysis after a single electrophoretic step, cDNAs made from these templates were 20-40% cross-contaminated by hybridisation analysis. This cross-contamination is presumably due to incomplete separation of the two mRNAs caused by heterogeneity in the poly(A) content of each. To improve mRNA separation, and thus the specificity of the cDNAs, we subjected each globin mRNA to two electrophoretic procedures (Fig. 1). The  $\alpha$  and  $\beta$  cDNAs made from these purified templates were then hybridised to increasing concentrations of mRNAs (Fig. 2). The percentage specificity of a cDNA was calculated by subtracting from 1 the ratio of the amount of homologous to heterologous mRNA needed to half-saturate that probe and multiplying the answer by 100 (refs 12, 13). Heterologous and homologous



**Fig. 1** Separation of  $\alpha$  and  $\beta$  globin mRNAs by polyacrylamide gel electrophoresis in formamide. Total cellular RNA and polyosomal RNA of reticulocytes were isolated<sup>21,22</sup> and separated into poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions by oligo(dT)-cellulose chromatography<sup>23</sup>. Poly(A)<sup>-</sup> fractions were chromatographed three or four times to remove traces of poly(A)<sup>+</sup> RNA. Poly(A)<sup>+</sup> RNA was subjected to electrophoresis on 5% polyacrylamide cylindrical gels in 98% formamide-20 mM Na barbital and 20 mM NaCl at 3.5 mA per gel at 24 °C for 2-3 h<sup>7,12</sup>. Globin mRNAs were located by scanning unstained gels at 260 nm and gel pieces containing the  $\alpha$  and  $\beta$  mRNAs were excised and incorporated with the same polarity into separate 7% polyacrylamide gels. After a second electrophoresis at 3.5 mA per gel for 3-4.5 h at 24 °C, the mRNAs were again located by ultraviolet scanning and the pertinent gel fractions were excised. In some experiments, RNA was eluted electrophoretically into dialysis bags<sup>7</sup>. The eluate was extracted with phenol, centrifuged to remove gel particles, re-extracted twice with chloroform and made 70% in ethanol to precipitate the mRNA. In recent experiments a different procedure which resulted in a twofold increase in yield was used. The gel pieces were driven through a 25-gauge needle with 2-3 volumes of 7 M urea, 0.35 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2% SDS. The resulting suspension was agitated at 45 °C for 2 h, underlayered with 60% glycerol, and centrifuged to remove gel pieces. After the aqueous layer was extracted twice with phenol and twice with chloroform, the RNA was precipitated with ethanol and purified by oligo(dT)-cellulose chromatography. (J. Ross, personal communication). *a*, Poly(A)<sup>+</sup> RNA of C57 Bl/6J mouse reticulocytes. After the 5% gel electrophoresis (left), each mRNA was excised and rerun in a 7% gel (right). mRNAs were excised as indicated by the vertical lines. *b*, Reticulocyte poly(A)<sup>+</sup> RNA of sheep homozygous for  $\beta$  globin after 5% gel electrophoresis.



**Fig. 2** Hybridisation of  $\alpha$  and  $\beta$  <sup>3</sup>H cDNAs with purified  $\alpha$  and  $\beta$  mRNA templates. <sup>3</sup>H-cDNAs were synthesised as reported<sup>24</sup> except the 100- $\mu$ l reaction mixture contained 100  $\mu$ g ml<sup>-1</sup> actinomycin D, 400  $\mu$ M each of dATP, dTTP, dCTP, and 100  $\mu$ M <sup>3</sup>H-dGTP (10 Ci mmol<sup>-1</sup>); 1-2  $\mu$ g RNA template, 0.125  $\mu$ g oligo(dT) per  $\mu$ g mRNA, and 300 units per ml AMV reverse transcriptase (from NCI). After incubation at 37 °C for 1 h, 50  $\mu$ g tRNA was added, the mixture extracted with phenol, and cDNA was precipitated with ethanol.  $\alpha$  and  $\beta$  cDNAs were treated for 24 h at 37 °C in 0.3 ml of 0.3 M NaOH, neutralised with 0.5 M HCl, applied to hydroxylapatite in 0.05 M Na phosphate buffer (pH 6.8) at 60 °C, and eluted in 0.14 M Na phosphate buffer (pH 6.8). The cDNAs (5-10S) were further purified by centrifugation in 10%-30% sucrose gradients (0.1 M NaCl; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.5% sodium dodecyl sulphate (SDS)) at 94,000g and 24 °C for 20 h and then precipitated with ethanol. Hybridisations of <sup>3</sup>H-cDNA to RNA were performed in sealed siliconised capillary tubes using a buffer containing 0.3 M NaCl, 30 mM Tris-HCl (pH 7), 2 mM EDTA, 0.1% SDS, and 40  $\mu$ g per ml *Escherichia coli* tRNA. Varying amounts of sample RNA and a constant amount of cDNA (500 c.p.m.) were diluted to 18  $\mu$ l, heated to 100 °C for 90 s, and then incubated 16-18 h at 68-70 °C. One-half of each sample was digested with S<sub>1</sub> nuclease, and the other half was an undigested control<sup>25</sup>. Both samples were then precipitated with 7.5% trichloroacetic acid on to S&S nitrocellulose filters. The filters were dissolved with 0.5 M HCl at 100 °C followed by ethyl acetate at 24 °C, and Hydromix was added before counting. Half of the difference in hybridisation between zero and saturating RNA inputs is defined as half-hybridisation. Specificity was calculated as described in the text. *a*, Rabbit  $\alpha$  cDNA = 94%; *b*, rabbit  $\beta$  cDNA = 94%; *c*, mouse  $\alpha$  cDNA = 97%; *d*, mouse  $\beta$  cDNA = 94%;  $\circ$ ,  $\alpha$  mRNA;  $\bullet$ ,  $\beta$  mRNA.

hybrids had identical thermal elution profiles ( $\pm 1^\circ$ ) indicating little or no mismatching of sequence and suggesting that heterologous hybrids are due to residual contamination rather than cross-hybridisation<sup>13</sup>. Alternatively, calculation of cDNA specificity by analysis of the shapes of curves seen in heterologous hybridisations suggests that residual contamination of the  $\beta$  cDNA (Fig. 2*b, d*) may be as high as 15-20%, while that of  $\alpha$  cDNA (Fig. 2*a, c*) is near 0. But, since the contamination of  $\beta$  mRNA with  $\alpha$  mRNA in Fig. 2*a* and *c* is no greater than 6% and 3%, respectively, this method of estimation may be invalid.

We have used the appropriate cDNA probes to measure the  $\alpha/\beta$  RNA ratios in reticulocytes of rabbits, mice, and sheep. Typical hybridisation results are shown in Fig. 3, and the data are summarised in Table 1. In our experiments we have attempted to avoid several potential pitfalls. First, our cDNA probes, while up to 97% specific, have residual contamination. The presence of such contamination becomes more critical as one measures smaller differences in  $\alpha/\beta$  mRNA ratio. Several assays using probes varying in specificity from 83% to 97% have yielded similar  $\alpha/\beta$  RNA ratios in rabbit and mouse reticulocytes (Table 1). Furthermore, reconstruction experiments in

Table 1 Ratios of  $\alpha$ -to  $\beta$ -globin RNA in total and polysomal RNA of rabbit, mouse and sheep erythrocytes

| cDNAs  | Expt no. | Minimum* specificity (%) |              | Total cellular RNA | $\alpha/\beta$ RNA† Polysomal RNA |                      |                      |
|--------|----------|--------------------------|--------------|--------------------|-----------------------------------|----------------------|----------------------|
|        |          | $\alpha$ cDNA            | $\beta$ cDNA |                    | Unfractionated                    | Poly(A) <sup>+</sup> | Poly(A) <sup>-</sup> |
| Rabbit | 1        | 94                       | 94           | —                  | 1.23                              | —                    | —                    |
|        | 2        | 86                       | 90           | 1.25               | 1.38                              | —                    | —                    |
|        | 3        | 85                       | 83           | 1.36               | 1.26                              | —                    | —                    |
| Mouse  | 1        | 97                       | 89           | 1.28               | 1.44                              | 1.43                 | 1.45                 |
|        | 2        | 97                       | 94           | 1.24               | 1.29                              | —                    | —                    |
| Sheep  | 1        | 90                       | 85           | 0.98               | 1.24                              | 1.26                 | 0.87                 |
|        |          |                          |              | 0.85               | 1.45                              | 0.92                 | 1.38                 |
|        |          |                          |              |                    | 1.29                              | 0.81                 | 1.36                 |

Ratios were calculated from half-saturation values using  $\alpha$  and  $\beta$  cDNA probes of indicated specificity. Each experiment contains data obtained with a different pair of cDNA probes and mRNA templates.

\*See text for method of calculation.

†For method of calculation of  $\alpha/\beta$  RNA ratios, see legend for Fig. 3.

which varying template mixtures were used demonstrated that differences in  $\alpha/\beta$  mRNA ratios of 10% or more could be detected. Second, to measure molar RNA ratios while controlling for known mRNA and potential cDNA size differences, we have determined hybridisation ratios with template standards. These ratios (between 0.9 and 1.1) were used to correct observed hybridisation ratios in test samples.

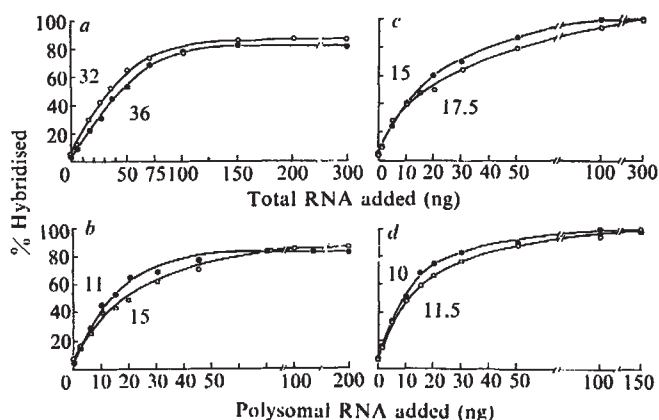
In rabbit reticulocytes, molar ratios of  $\alpha$  to  $\beta$  mRNA average 1.3 in total and polysomal RNA (Table 1). Similar  $\alpha/\beta$  polysomal RNA ratios were obtained using three different sets of cDNA probes with specificities ranging from 83% to 94%. While the ratios of  $\alpha/\beta$  mRNA in total and polysomal RNA were equal, the  $\alpha$  and  $\beta$  mRNA concentrations (calculated by comparing template and sample RNA amounts needed for half-hybridisation) were greater in polysomal ( $\alpha, \beta = 1.5\%, 1.2\%$ ) than in total RNA ( $\alpha, \beta = 1.1\%, 0.8\%$ ) because of the presence of 4S RNA in total RNA samples. When polysomal RNA was fractionated by oligo(dT)-cellulose chromatography, both poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions (see legend to Fig. 1) had the same  $\alpha/\beta$  ratio, and 95% of each mRNA was in the poly(A)<sup>+</sup> fraction. By subtraction of the  $\alpha$  and  $\beta$  globin mRNA amounts in polysomal RNA from those in total RNA, we calculate that 9% of  $\alpha$  mRNA and less than 1% of  $\beta$  mRNA are present in non-polysomal RNA of rabbit reticulocytes, in agreement with previous estimates<sup>14</sup>.

In C57 B1/6J mice, both the total and polysomal RNA of reticulocytes have average  $\alpha/\beta$  RNA ratios of approximately 1.3 (Table 1, Fig. 3c and d). Again, globin mRNA concentrations are higher in polysomal ( $\alpha, \beta = 1.6\%, 1.3\%$ ) than in total RNA ( $\alpha, \beta = 1.2\%, 0.9\%$ ). Note, however, that poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions of mouse polysomal RNA have different  $\alpha/\beta$  RNA ratios (1.26 and 0.87, respectively). Also, the  $\alpha/\beta$  RNA ratio in polysomal RNA calculated from the poly(A)<sup>+</sup> and the poly(A)<sup>-</sup> data agrees closely with the observed ratio (1.20 compared with 1.24). Since the ratio of  $\alpha/\beta$  in the poly(A)<sup>+</sup> RNA is 45% greater than in the poly(A)<sup>-</sup> fraction,  $\alpha$  mRNA may contain greater average poly(A) length than  $\beta$  mRNA in these mouse samples. Mouse and human mixed globin mRNAs are known to have different size classes of poly(A)<sup>15-17</sup>. The existence of different  $\alpha/\beta$  RNA ratios in poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions of mouse polysomal RNA suggests that such poly(A) size classes may have different distributions in  $\alpha$  and  $\beta$  mRNAs. Measurement of poly(A) length in the poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions should verify these differences.

Results obtained from reticulocyte RNA of homozygous  $\beta^B$  sheep contrast with the rabbit and mouse results. First, while the  $\alpha/\beta$  ratio in reticulocyte polysomal RNA of sheep is similar to that of rabbit and mouse, about 1.4, the  $\alpha/\beta$

RNA ratio in total RNA is only 0.9 (Table 1, Fig. 3a, b). In addition,  $\alpha$  and  $\beta$  mRNAs make up 1.8% and 1.5% of polysomal RNA, but only 0.62% and 0.70% of total RNA, respectively. Second, the results obtained by assays of poly(A)<sup>+</sup> RNA differ greatly from those obtained for unfractionated polysomal RNA. By gel electrophoresis, the  $\alpha/\beta$  RNA ratio of sheep poly(A)<sup>+</sup> RNA is about 0.5, or half that of rabbit and mouse poly(A)<sup>+</sup> RNA, implying an excess of  $\beta$  mRNA in poly(A)<sup>+</sup> RNA of sheep (Fig. 1b). By hybridisation analysis, an excess of  $\beta$  RNA was found in polysomal poly(A)<sup>+</sup> RNA ( $\alpha/\beta = 0.86$ ), while an excess of  $\alpha$  RNA was present in polysomal poly(A)<sup>-</sup> RNA ( $\alpha/\beta = 1.37$ ). But, the ratio of  $\alpha/\beta$  RNA in polysomal RNA calculated from the poly(A)<sup>+</sup> and poly(A)<sup>-</sup> data does not agree well with the observed polysomal  $\alpha/\beta$  ratio (1.0 compared with 1.29), possibly due to the lower specificities of the sheep cDNA probes. It is unlikely that the observed  $\beta$  mRNA excess in poly(A)<sup>+</sup> polysomal RNA of these  $\beta^B$  sheep is  $\beta^C$  mRNA, since Benz has demonstrated with  $\beta^C$  specific cDNA that  $\beta^B$  homozygotes essentially lack  $\beta^C$  genes<sup>18</sup>. The proportions of sheep mRNAs in polysomal poly(A)<sup>-</sup> RNA were greater than those observed in rabbit or mouse reticulocytes, but the percentages varied depend-

Fig. 3 Hybridisation of reticulocyte total and polysomal RNA to  $\alpha$  (●) and  $\beta$  (○) cDNAs of sheep (a, b) and mouse (c, d). Relative concentrations of  $\alpha$  and  $\beta$  mRNA are determined by comparing amounts of sample RNA needed to half-saturate each cDNA probe. Using homologous templates as reference standards, the amounts needed to half-saturate each set of  $\alpha$  and  $\beta$  cDNAs usually differed by less than 10% ( $\alpha, \beta = 0.27, 0.28$  and  $0.35, 0.33$  ng, respectively, for sheep and mouse cDNAs shown). The ratios obtained using template standards were used to correct observed sample ratios. For example in c,  $\alpha/\beta = (17.5/15) \times (0.35/0.33) = 1.24$ .





ing on whether the observed concentration of globin mRNAs in total polysomal RNA or the calculated concentration (sum of poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RNA fractions) was used (poly(A)<sup>-</sup>  $\alpha$  = 41.25%,  $\beta$  = 13.17%). These results suggest a substantial difference in the  $\alpha/\beta$  RNA ratio between the poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions in these sheep samples.

Since poly(A) length may affect mRNA stability and/or transport from nucleus to cytoplasm, differential adenylation of globin mRNAs could be important in balancing globin chain synthesis<sup>18,20</sup>. Thus, if sheep  $\alpha$  mRNA has a shorter average poly(A) length than  $\beta$  mRNA, as suggested by our data, any pool of  $\alpha$  mRNA not associated with ribosomes in early erythroid cells could be degraded at a faster rate than  $\beta$  mRNA, thereby accounting for the low  $\alpha/\beta$  ratio observed in total RNA of sheep reticulocytes (Table 1, Fig. 3a).

Thus, our data indicate that  $\alpha/\beta$  mRNA ratios in poly(A)<sup>+</sup> RNA may not reflect the actual  $\alpha/\beta$  ratio in unfractionated total or polysomal RNA. In this regard we have recently re-examined the accumulation of globin mRNAs in erythropoietic mouse spleen cells. Gel analyses of globin mRNAs in poly(A)<sup>+</sup> total RNA of spleen had yielded  $\alpha/\beta$  ratios of 0.55–0.8 at different times after phenylhydrazine injection<sup>9</sup>. By hybridisation, we now find a constant  $\alpha/\beta$  RNA ratio of 1.25 in unfractionated total RNA at all times. In contrast, poly(A)<sup>+</sup> total RNA of spleen cells have  $\alpha/\beta$  RNA ratios by hybridisation which parallel those obtained previously by gel analysis (J. A. P., in preparation). Thus, while a changing  $\alpha/\beta$  ratio and  $\beta$  excess in poly(A)<sup>+</sup>-selected RNA is seen with both methods, in total spleen RNA an  $\alpha$  RNA excess is observed using specific cDNA probes.

In addition, by hybridisation we have found  $\alpha/\beta$  RNA ratios in polysomes of 1.2–1.4 in rabbit, mouse and sheep reticulocytes in agreement with theoretical predictions of functional  $\alpha/\beta$  mRNA ratios<sup>4,5</sup>. Our results suggest that balanced synthesis of  $\alpha$  and  $\beta$  chains in these species depends on an excess of  $\alpha$  mRNA on polysomes, regardless of the relative poly(A) length of the two messages. In these animals, the  $\alpha/\beta$  mRNA ratios of 1.2 to 1.4 differ significantly from the  $\alpha/\beta$  gene ratios. Thus, a further regulation of gene expression presumably occurs at transcription and/or mRNA processing.

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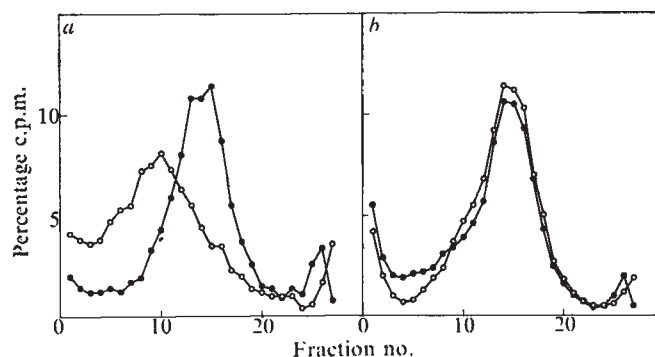
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## Evidence for alkali-sensitive linkers in DNA of African green monkey kidney cells

MITOCHONDRIAL DNA possesses ribonucleotide linkers<sup>1–3</sup> and although double-stranded eukaryotic chromosomal DNA seems to be continuous throughout the length of the chromosome<sup>4</sup> it is possible that single-stranded DNA possesses physical (nicks) or chemical (non-deoxynucleotide linkers) discontinuities. To distinguish between an *in vivo* nick and a nick introduced by shear forces during DNA extraction is very difficult with the presently available techniques. The detection of non-deoxynucleotide linkers is, however feasible, providing that denatured DNA can be sedimented in a chemical environment in which the linker is not destroyed. In these conditions, the comparison between sedimentation profiles of DNA, previously treated or not with a reagent that attacks the linker, may reveal the discontinuity. An obvious such reagent is a strong base, thus precluding the use of alkaline gradients in these experiments, and so we have attempted to use sucrose gradients in non-aqueous formamide. In addition, the



**Fig. 1** Alkali-sensitive sites in green monkey kidney cell DNA. The cells were grown in glass Petri dishes, in Dulbecco's modified medium containing 10% calf serum, in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C, and labelled for 8 h with <sup>3</sup>H-thymidine (2  $\mu$ Ci ml<sup>-1</sup>). The cells were washed with saline-EDTA (100 mM NaCl, 10 mM EDTA, pH 8.0) and incubated with 2.0 ml of 0.5% Triton X-100 in saline EDTA for 1 min. The nuclei remained attached to the bottom of the plate and were lysed by incubating for 30 min at 37 °C with 0.75% Sarkosyl, 1 M NaCl, 10 mM EDTA, pH 8.0. The proteins were extracted by mixing the lysate gently with equal volume of 80% phenol at 4 °C. The aqueous phase was separated by centrifugation and gently mixed again with equal volume of chloroform-isoamyl alcohol (20:5, v/v). The final aqueous phase was dialysed overnight against 2 l of 10 mM Tris-HCl, pH 7.5. Aliquots (0.25 ml) of DNA were layered on the top of 4.0-ml gradients which were 0–15% sucrose (w/v) in formamide. Centrifugation was at 32,000 r.p.m. at 30 °C in an SW-50.1 rotor (Spinco) for 4 h. A total of 26–27 equal volume fractions were dripped on to strips of Whatman No. 17 paper and radioactivity was measured as described elsewhere<sup>14</sup>. **a**, Percentage <sup>3</sup>H-DNA from monkey cells; **b**, percentage <sup>3</sup>H-DNA from T4 phage; ○, 100  $\mu$ l of DNA solution were mixed with 50  $\mu$ l of 1 M Tris-HCl, pH 7.0 and 100  $\mu$ l of 0.225 M NaCl, before layering on the gradients; ●, 100  $\mu$ l of the DNA solution were mixed with 50  $\mu$ l of 0.45 M NaOH, incubated for 10 min at 20 °C, mixed with 50  $\mu$ l of 1 M Tris-HCl followed by 50  $\mu$ l of 0.45 M HCl, and then layered on the gradients. The number average molecular weights ( $M_n$ ) were calculated as described elsewhere<sup>14</sup>, except that in Studier's expression the exponent 0.400 was replaced by 0.312 which was better fitted to our conditions, as determined with T4 and  $\lambda$ DNA. **a**,  $M_n$  (○) = 68.2;  $M_n$  (●) = 45.6; **b**,  $M_n$  (○) = 46.2;  $M_n$  (●) = 49.8.



DNA had to be extracted by a very gentle method in order to keep its average molecular weight in the range of  $50\text{--}80 \times 10^6$ . Using these conditions, we were able to detect alkali-sensitive linkers in chromosomal DNA of green monkey kidney cells.

A typical result is shown in Fig. 1a. DNA which has been sedimented without any previous treatment has a number average molecular weight ( $M_n$ ) equal to  $68.2 \times 10^6$  daltons. Exposure of DNA to 0.15 M NaOH for 5 min at 20 °C, before sedimentation causes  $M_n$  to drop to  $45.6 \times 10^6$  daltons and the profile becomes less heterogeneous. Figure 1b shows that the same treatment on T4 DNA produces no change in the sedimentation profile; T7 DNA displays exactly the same behaviour (results not shown). The results of several experiments similar to that shown in Fig. 1a are given in Table 1. The average number of alkali-sensitive linkers for five different determinations (a–e) was  $0.87 \pm 0.29$  per  $10^8$  daltons. This means that an average of one linker is found for every  $115 \times 10^6$  daltons. In one case (Table 1e) the NaOH treatment was more drastic, without introducing comparatively more breaks, therefore, the more gentle reaction condition seems to give a full account of the linker disruption. In two other experiments (Table 1f,g) DNA was pulse-labelled for 25 min and the number of breaks observed was larger than in DNA labelled for several hours; other experiments are needed to define whether these results reflect a modification of the frequency of linkers as the DNA matures.

In opposition to the linker hypothesis, it could be argued that formamide does not render the DNA completely denatured, and that this is only accomplished after treatment with NaOH. This seems not to be the case, however, since T4 DNA displays the same sedimentation profile with or without NaOH treatment. Further evidence that DNA is already denatured before NaOH treatment is given by the experiment of Fig. 2. In this experiment, we intended to produce a DNA in which the opposite strands were labelled differently and had different sizes. If, on centrifugation in formamide, we obtained separation of the two sedimentation profiles we could conclude that the DNA was denatured. Cells were therefore prelabelled with  $^{14}\text{C}$ -thymidine for 48 h and then pulse-labelled with  $^3\text{H}$ -thymidine for 25 min. In these conditions, long strands of  $^{14}\text{C}$ -labelled parental DNA should be hydrogen bound to strands of  $^3\text{H}$ -labelled, newly synthesised DNA. This latter should be more heterogeneous in size, because it corresponds to growing strands of all sizes between zero and the size of the replicating unit<sup>5,6</sup> (about  $10^6$  daltons). Denaturation is thus denoted in this experiment by the separation of the two profiles, in a situation essentially identical to that obtained in alkaline sucrose gradients<sup>6</sup>.

We conclude from these results that the DNA molecules from African green monkey kidney cells contain linkers that are sensitive to alkali and that they are, on average, located  $115 \times 10^6$  daltons apart. The nature of these linkers is now being investigated and two main possibilities are

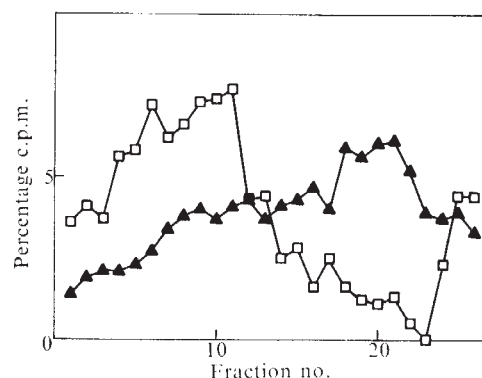


Fig. 2 Strand separation in sucrose-formamide gradients. The cells were incubated in  $^{14}\text{C}$ -thymidine ( $0.1 \mu\text{Ci ml}^{-1}$ ) for 48 h. The medium was withdrawn and the cells incubated for 25 min in  $50 \mu\text{Ci ml}^{-1}$   $^3\text{H}$ -thymidine. The DNA was extracted, centrifuged, fractionated and counted as described in the legend of Figure 1.  $\square$ , Percentage of  $^{14}\text{C}$ -DNA,  $M_n = 62.8$ ;  $\blacktriangle$ , percentage of  $^3\text{H}$ -DNA,  $M_n = 20.5$ .

being pursued, namely, a ribonucleotide linker and an apurinic and/or apyrimidinic site.

Ribonucleotides have been detected in mature closed-circular mitochondrial DNA<sup>1–3</sup>. Their presence could be envisaged as due to insertion into DNA during replication by a relatively non-stringent DNA polymerase or that they are remnants of RNA primers which have not been completely excised. Substantial evidence has been presented from several systems that an RNA polymerase product provides the 3'-hydroxy primer required by all known DNA polymerases<sup>7–9</sup>. Apurinic sites are known to be formed spontaneously in DNA at a rate corresponding to  $5 \times 10^{-2}$  depurinations per  $10^8$  daltons per h at 37 °C (ref. 10). Table 1 shows that the linkers are already present at a frequency of 1.5 per  $10^8$  daltons in short pulse-labelled DNA, therefore, apurinic/apyrimidinic sites could only have been introduced catalytically, such as by a base alkylation mechanism.

Several laboratories have reported that treatment of cell lysates with proteases before centrifugation in alkaline sucrose gradients decreased the size of the DNA molecules<sup>11–13</sup>. After the treatment, single-strand pieces of  $60 \times 10^6$ ,  $45 \times 10^6$  and  $6 \times 10^6$  daltons were observed in CHO (ref. 11), mouse L cells<sup>12</sup> and Ehrlich ascites tumour cells<sup>13</sup>, respectively. The connection between these results and ours is not clear as yet. A preliminary experiment in which NaOH was replaced by proteinase K did not show any reduction in the molecular weight. It is possible that the protease treatment of the cell lysates removed protein from the DNA rendering the linkers accessible to the alkali. In our experiments, these proteins would have been extracted by the phenol-chloroform treatment and so

Table 1 Number of single-strand breaks produced in DNA on treatment with sodium hydroxide

| Experiment | $M_n$ untreated | $M_n$ treated | No. of breaks per $10^8$ daltons | Treatment                  |
|------------|-----------------|---------------|----------------------------------|----------------------------|
| a          | 71.9            | 49.7          | 0.62                             | 0.15 M NaOH, 10 min, 20 °C |
| b          | 66.7            | 42.4          | 0.86                             | 0.15 M NaOH, 5 min, 20 °C  |
| c          | 78.3            | 38.4          | 1.32                             | 0.15 M NaOH, 5 min, 20 °C  |
| d          | 68.2            | 45.6          | 0.73                             | 0.15 M NaOH, 5 min, 20 °C  |
| e          | 71.9            | 45.2          | 0.82                             | 0.50 M NaOH, 1 h, 37 °C    |
| f          | 42.7            | 26.7          | 1.40                             | 0.15 M NaOH, 10 min, 20 °C |
| g          | 42.7            | 25.3          | 1.61                             | 0.50 M NaOH, 1 h, 37 °C    |

a–e, DNA was labelled with radioactive thymidine for periods varying from 8 to 48 h. In f and g DNA was pulse-labelled for 25 min only. Determination of  $M_n$  is described in the legend to Fig. 1. Alkali treatment varied as indicated. The number of single-strand breaks was calculated using the expression  $(M_n \text{ treated}/M_n \text{ untreated}) - 1$ . The values were normalised to  $10^8$  daltons.

the alkali-sensitive sites would already be accessible to attack.

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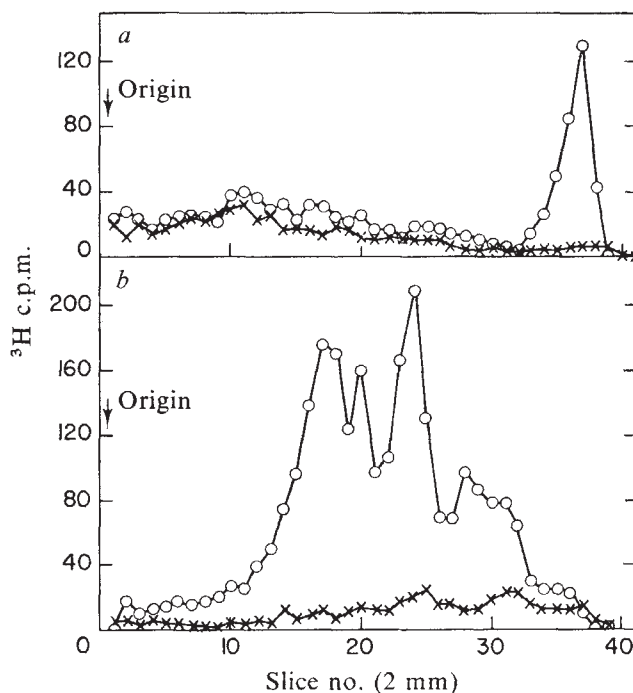
## Chloramphenicol-sensitive labelling of protein in microsomes of *Neurospora crassa*

SEVERAL peptides are synthesised on mitochondrial chloramphenicol-sensitive ribosomes<sup>1</sup>. In the presence of cycloheximide ( $\sim 200 \mu\text{g ml}^{-1}$ ), amino acids are incorporated exclusively into mitochondrial proteins in *Neurospora crassa*<sup>2</sup> and the flight muscle of *Locusta migratoria*<sup>3</sup>, leading to the generally accepted idea that mitochondrially synthesised proteins remain in the mitochondria. It is possible, however, that the high concentration of cycloheximide used in those experiments could inhibit any potential export of such proteins to other parts of the cell, particularly since the drug has been shown to affect several vital cellular processes independently of its effect on protein synthesis; for example, it stimulates the catabolism of nucleic acids and decreases intracellular ATP (ref. 4). This may explain some published indications of export of mitochondrially synthesised proteins in experiments in which cycloheximide was not used, such as the continued synthesis of myelin proteolipid in crude mitochondrial fractions of rat brain homogenates treated with RNase<sup>5</sup>; and the cytoplasmic location of the mutation in a line of *Zea mays* responsible for the susceptibility of its root plasma membrane  $\text{K}^+$ -stimulated ATPase to the toxin of *Helminthosporium maydis* race T.<sup>6</sup> We describe here observations on the incorporation of radioactivity into membranes of the post-mitochondrial supernatant of homogenates of *N. crassa* which had been pulsed with radioactive amino acids in the presence of cycloheximide ( $25 \mu\text{g ml}^{-1}$ ) then grown for 2 h in drug- and label-free medium. We have been unable to account for this solely on the basis of mitochondrial fragmentation. Of the membranes present in the postmitochondrial supernatant, we have investigated in detail a microsomal fraction enriched for endoplasmic reticulum, as a first step in determining the nature of this incorporation.

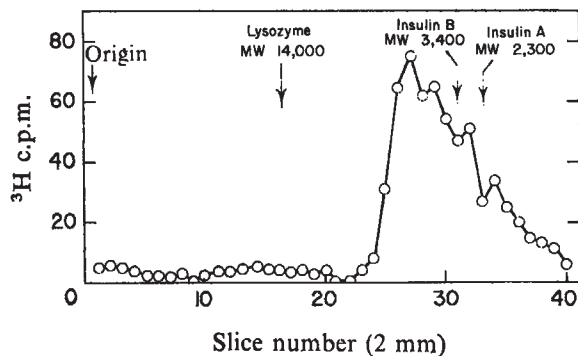
*N. crassa* 74A was cultured and pulse-chase labelled with amino acids in the presence of selective inhibitors of pro-

tein synthesis. Microsomes and mitochondria were isolated as described in the legend to Fig. 1, and the two fractions characterised by marker enzyme assays. The microsomal fraction was enriched 10-fold over the mitochondria in glucose-6-phosphatase activity measured according to de Duve *et al.*<sup>10</sup>, and no haem *a* was detectable in reduced minus oxidised difference spectra<sup>11</sup>. The assays indicated that the microsomes were enriched in endoplasmic reticulum and virtually devoid of mitochondrial inner membrane.

Four proteins whose synthesis is chloramphenicol-sensitive are normally obtained from pulse-labelled *N. crassa* mitochondria. Their molecular weights are roughly 10,000, 20,000, 30,000 and 40,000. The 10,000 molecular



**Fig. 1** Distribution of radioactivity in *a*, microsomes and *b*, mitochondria. *N. crassa* was labelled as described previously<sup>7</sup>, except that  $200 \mu\text{Ci}$  of  $[\text{G-}^3\text{H}]\text{L-phenylalanine}$  (Schwarz-Mann,  $10 \text{ Ci mmol}^{-1}$ ), per  $500 \text{ ml}$  of medium was routinely used in place of leucine as the labelling agent. In one series of experiments the cultures were labelled with  $500 \mu\text{Ci } ^3\text{H-amino acid mixture}$  (Amersham/Searle code TRK.440) per  $600 \text{ ml}$  of medium. The cells were labelled in the presence of cycloheximide ( $25 \mu\text{g ml}^{-1}$ ) or cycloheximide ( $25 \mu\text{g ml}^{-1}$ ) plus chloramphenicol ( $4 \text{ mg ml}^{-1}$ ). Mycelial pads were collected by vacuum filtration and disrupted in a mill (B. Braun, Melsungen) with  $0.5\text{-mm}$  glass beads in the following buffer:  $0.5 \text{ M}$  sucrose,  $1 \text{ mM}$  EDTA, and  $50 \text{ mM}$  Tris-HCl,  $\text{pH } 7.5$ . To inhibit protease activity,  $0.5 \text{ mM}$  phenylmethylsulphonyl fluoride was added to all buffers. Nuclei and the bulk of the cell wall were removed by centrifugation at  $2,000g$  for  $10 \text{ min}$ , and a heavy mitochondrial fraction was obtained by centrifugation of the supernatant at  $8,000g$  for  $15 \text{ min}$ . The mitochondria were washed by homogenising in the same buffer, and repeating the above centrifugations. Light mitochondria and lysosomes were removed from the postmitochondrial supernatant by centrifugation at  $50,000g$  for  $30 \text{ min}$ , and discarded. Microsomes were then prepared by centrifuging the supernatant at  $105,000g$  for  $1 \text{ h}$ . The pellet was washed by resuspension in the same buffer, followed by centrifugations at  $50,000g$  and  $100,000g$ . All operations were carried out at  $0-4^\circ\text{C}$ . Protein was measured by the method of Lowry *et al.*<sup>8</sup>. Microsomes and mitochondria were assayed by SDS-polyacrylamide gel electrophoresis in sodium dodecyl sulphate, using  $11.5\%$  gels as described by Fairbanks *et al.*<sup>9</sup>, as modified by Lansman *et al.*<sup>7</sup>. The radioactivity in  $2\text{-mm}$  gel slices was measured by liquid scintillation counting after swelling in  $0.4 \text{ ml}$  NCS Tissue solubiliser (Amersham/Searle) for  $2.5 \text{ h}$  at  $45^\circ\text{C}$  followed by addition of toluene plus Omnifluor (Packard). *a*,  $100 \mu\text{g}$  sample of microsomes; *b*,  $100\text{-}\mu\text{g}$  sample of mitochondria;  $\circ$ , cycloheximide;  $\times$ , cycloheximide plus chloramphenicol.



**Fig. 2** Determination of the apparent molecular weight of CSMP. Microsomes (650  $\mu\text{g}$ ), labelled in the presence of cycloheximide as described in Fig. 1, were extracted with chloroform-methanol (2:1) centrifuged at 5,000g for 10 min and the supernatant dried under nitrogen. The residue was electrophoresed in SDS-urea polyacrylamide gels according to Swank and Munkres<sup>12</sup>. Radioactivity was determined as described in Fig. 1. The position of markers was determined after staining with Coomassie Blue<sup>12</sup>.

weight (MW) peak disappears from mitochondria after a 2-h chase period<sup>7</sup>. In experiments reported here, equal quantities of mitochondrial and microsomal protein (100  $\mu\text{g}$  per gel) from cells pulse-chase labelled in the presence of cycloheximide or cycloheximide plus chloramphenicol were compared by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The distribution of radioactivity in mitochondria and in microsomes from cells pulse-chase labelled in the presence of cycloheximide alone showed distinct differences (Fig. 1). The mitochondria had little protein of MW about 10,000 and none smaller than this, as expected for *N. crassa* mitochondria labelled in this manner<sup>7</sup>. In contrast, the majority of the radioactivity in the microsomes appeared in a low MW protein which was smaller than the 10,000 MW protein seen in mitochondria after a pulse-label experiment, and is therefore unlikely to be derived from mitochondrial membrane fragments. The synthesis of the microsomal protein was reproducibly sensitive to chloramphenicol. The apparent MW of this chloramphenicol-sensitive microsomal protein (CSMP) as determined by polyacrylamide gel electrophoresis in the presence of SDS-urea was 5,000 to 8,000 (Fig. 2). CSMP was readily extractable from microsomes with 20 volumes of 80% acetone or with chloroform-methanol (2:1), the latter property suggesting it is a proteolipid.

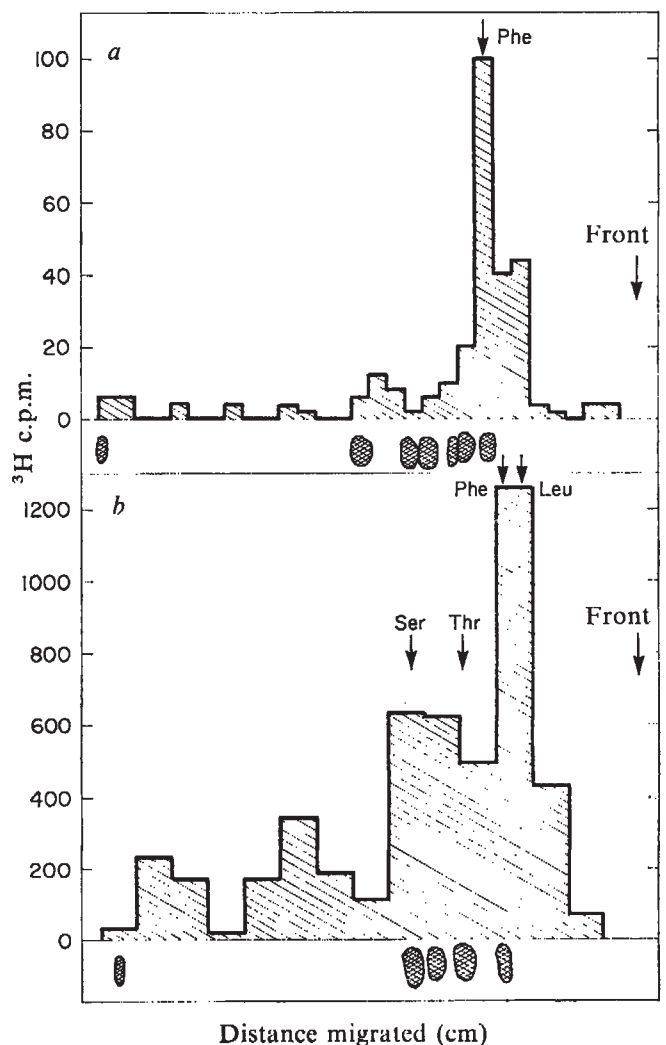
Saponification of chloroform-methanol extracts of microsomes labelled in the presence of cycloheximide, which should release any amino acids present as amino-lipids, revealed negligible amounts of radioactivity recoverable as amino acids, suggesting that the bulk of the radioactivity was incorporated into protein. Only 6% of the radioactivity was recovered in petroleum ether extracts after saponification of  $^3\text{H}$ -phenylalanine-labelled CSMP, indicating a low level of amino acid conversion to lipid. Total acid hydrolysis of CSMP was obtained only after saponification, however, suggesting that the peptide chain may have been protected to some degree by lipids. Labelled phenylalanine (as assayed by thin-layer chromatography) was recovered from total acid hydrolysates of CSMP isolated from cells pulse-labelled with  $^3\text{H}$ -phenylalanine (Fig. 3a). Several different amino acids were detected by ninhydrin visualisation and radioactivity in total hydrolysates of CSMP isolated from cells labelled with a mixture of  $^3\text{H}$ -amino acids (Fig. 3b).

Thin-layer chromatography of chloroform-methanol extracts of microsomes or mitochondria in a system that separates a variety of lipids gave a band of radioactivity whose synthesis was chloramphenicol sensitive. This band

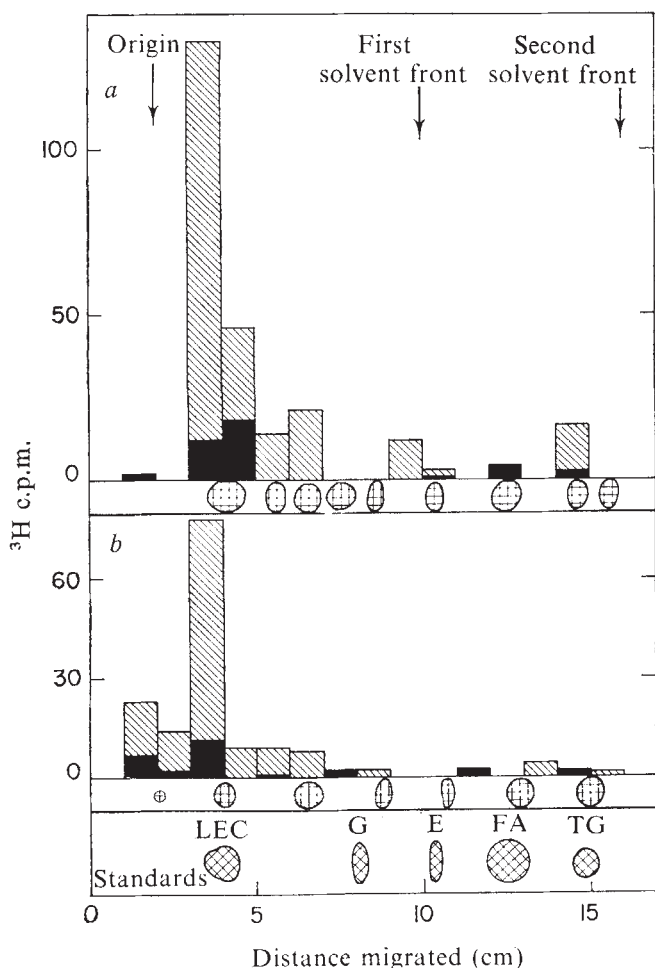
migrated with an  $R_f$  similar to that reported for a low molecular weight proteolipid synthesised by isolated rat liver mitochondria<sup>15,16</sup>, and which is similar to the  $R_f$  of phosphatidyl choline (Fig. 4a and b). The acid hydrolysis data described above, however, indicated that the radioactivity was recovered in amino acids, not in phosphatidyl choline. Phosphatidyl serine, the most common amino-lipid, migrates with an  $R_f$  close to 0.4 in the first developing system. Thus, the radioactivity in CSMP could not be due to label converted to phosphatidyl serine.

All the above data suggest very strongly that CSMP is indeed a protein, albeit with somewhat unusual properties.

**Fig. 3** Analysis of total hydrolysates of chloramphenicol-sensitive cycloheximide-insensitive material by thin-layer chromatography. *N. crassa* microsomes labelled in the presence of cycloheximide with  $^1\alpha$ , [ $^3\text{H}$ ] phenylalanine (see Fig. 2) or b,  $^3\text{H}$ -amino acid mixture (as described in the text) were extracted in chloroform-methanol (2:1) and dried under nitrogen. The dried residues were saponified in 95% methanol, 0.5 M NaOH at 30 °C for 15 h, acidified to pH 3 with 6 M HCl and extracted three times with 2 vol. petroleum ether which removed non-saponifiable lipids. The methanolic layer was neutralised, dried under  $\text{N}_2$  and hydrolysed in 6 M HCl at 110 °C for 72 h. HCl was removed in a vacuum desiccator over NaOH and concentrated  $\text{H}_2\text{SO}_4$ . The residues were dissolved in water and desalted, using a Dowex-2 (AG-2 X-10, BioRad) anion exchange resin (which also removed ethanolamine and choline)<sup>13</sup> followed by removal of HCl and acetic acid by vacuum desiccation as above. Amino acids were separated on silica gel plates (EM Laboratories), with ethanol-water (7:3), and visualised with ninhydrin (shown as cross-hatched spots below the graphs). The radioactivity was determined by liquid scintillation counting of 0.5-cm bands in Bray's solution<sup>14</sup>.







**Fig. 4** Thin-layer chromatography of chloroform-methanol extracts of subcellular fractions. *a*, Microsomes and *b*, mitochondria were isolated from *N. crassa* pulse-chase labelled in the presence of cycloheximide or of cycloheximide plus chloramphenicol. Membrane suspensions (146  $\mu\text{g}$  protein) were extracted with 1 ml of chloroform-methanol (2:1) at room temperature for 10 min followed by centrifugation at 5,000g for 10 min. The supernatants were dried under nitrogen, dissolved in a small volume of the same solvent, and separated by thin-layer chromatography on silica gel HR (Merck). Phospholipids and neutral lipids were separated by successive developments in chloroform-methanol-glacial acetic acid-water (65:25:8:4) (ref. 17) and then petroleum ether-diethyl ether-glacial acetic acid (75:25:1) (ref. 18). Lipids were visualised with iodine vapour (shown as cross-hatched spots below the graphs). Bands (0.5 cm) were scraped from the plate into counting vials, and radioactivity was determined by counting in toluene-liquifluor (New England Nuclear) using a Packard Tri-Carb liquid scintillation counter. Standards were: LEC, dipalmitoyl lecithin; G, gramicidin D; E, ergosterol; FA, oleic acid; TG, triolein. Cycloheximide, hatched; cycloheximide plus chloramphenicol, solid.

Purification of this protein is in progress. After gel filtration of the chloroform-methanol soluble fraction of *N. crassa* microsomes on LH-20 columns, a single radioactive ninhydrin-positive spot is observed on paper chromatography.

The appearance of the protein CSMP in the microsomal fraction could be explained in several ways. As shown above, it is unlikely to be due to simple cross-contamination from mitochondrial inner membranes. The results could be accounted for by CSMP escaping from the mitochondria during the isolation procedure, but a very hydrophobic protein such as this is unlikely to be removed from mitochondrial membranes in the isolation conditions used. It is unlikely that CSMP is the result of labelling of microsomal protein(s) by a non-ribosomal-dependent enzyme

system insensitive to cycloheximide but sensitive to chloramphenicol as has been reported for *in vitro* preparations of myelin<sup>19</sup>. In the conditions used for *in vitro* incorporation of radiolabelled amino acids into a myelin fraction<sup>19</sup>, no chloramphenicol-sensitive incorporation of  $^3\text{H}$ -phenylalanine by a 20,000g supernatant of an *N. crassa* homogenate was found. The only labelling observed was sensitive to cycloheximide and insensitive to chloramphenicol, suggesting normal *in vitro* cytoplasmic ribosomal-dependent incorporation of amino acids. These observations are in direct contrast to the *in vivo* labelling pattern of CSMP, supporting our contention that CSMP is synthesised on chloramphenicol-sensitive ribosomes and not by an unusual enzymatic reaction sensitive to chloramphenicol. It is possible that there are hitherto undescribed cycloheximide-insensitive, chloramphenicol-sensitive ribosomes outside the mitochondria. It is also possible that CSMP is a product of mitochondrial protein synthesis which has been processed during or after export. In summary, the evidence is most consistent with CSMP being synthesised on mitochondrial ribosomes and exported from the mitochondria.

Preliminary experiments with isolated rat hepatocytes have indicated that export of low molecular weight proteolipids from mitochondria to the endoplasmic reticulum and also to the plasma membrane occurred.

The role or roles of CSMP in the cell are unknown. Several investigators have concluded mitochondrial export to the cytoplasm must exist. Some of these reports suggest a repressor function for this protein. Barath and Kuntzel<sup>20</sup> observed that in the presence of chloramphenicol or ethidium bromide, an increased biosynthesis of certain nuclear gene products associated with the *Neurospora* mitochondrial transcriptional and translational apparatus occurred. A model was proposed for a mitochondrial repressor which regulates the synthesis of these proteins. Such a repressor has also been proposed by Edwards *et al.*<sup>21</sup> to explain a similar chloramphenicol effect on the induction of the cyanide-insensitive alternate oxidase of *Neurospora*, an enzyme that is synthesised in the cytoplasm. This repressor model could also explain the induction of the alternate oxidase in the mitochondrial mutants *mi-1* and *mi-3* of *Neurospora*<sup>22</sup>.

Export of mitochondrial protein could be the functional basis for a mitochondrial linkage to cellular transformation. This is suggested by the protection of BALB 3T3 cells from viral transformation by pretreatment with ethidium bromide<sup>23</sup>, by the close correlation of the severity of granulocytic leukaemia with the frequency of circular dimers of mitochondrial DNA in leukocytes<sup>24</sup> and by the distinct and specific changes in the synthesis of mitochondrial DNA in chick embryo fibroblasts transformed by Rous sarcoma virus<sup>25</sup>.

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## A cholesterol-isopropanol gel

In the course of measuring solubilities of cholesterol in pure and mixed solvents we have observed the formation of a transparent gel in the cholesterol+isopropanol system. Because of the historical importance of liquid crystals of cholesteryl esters<sup>1</sup> and the biological importance of cholesterol, we are reporting here some observations on the properties of this gel. Liquid crystals of cholesterol with fatty alkanols ( $C_{12}$ – $C_{18}$ ) are known<sup>2,3</sup>, but have not been reported for smaller alcohols. The transparent gel of cholesterol and isopropanol seems to be stable at 4 °C for concentrations of 4.6–5.3% (w/w) cholesterol. At higher concentrations and temperatures (up to 10.2% at 22 °C), the transparent gel can be observed for a day or more, then white 'snowflakes' appear throughout the gel and grow slowly into a translucent or opaque mass. On warming, the transparent gels liquify, and the translucent gels seem to go directly to the liquid state. The transparent gel is unstable to vigorous shaking; samples with less than 5.5% cholesterol liquify then return to the transparent gel state on standing at 4 °C. Samples with larger concentrations form the translucent or opaque state on shaking and do not return to the transparent state over a period of several weeks. Observations of the transparent gel under a polarising microscope at room temperature showed the sample to be mostly isotropic with some small points of optical activity.

Cholesterol (Fisher Certified Reagent Grade) originally with a melting point of 147–148 °C. After several recrystallisations from ethanol the melting point was 148–149 °C. Isopropanol (Matheson, Coleman and Bell Spectroquality) was stored over molecular sieves and twice distilled, retaining the middle one-third each time. In all of the measurements reported here, these purified materials were used, but very similar results were obtained with untreated materials. Samples were prepared by weight, sealed in glass ampoules under reduced pressure, warmed to dissolve the solid, and then allowed to stand without shaking in four different environments ranging from –10–22 °C, all with observed temperature fluctuations of about 1 °C. Four physical states were observed: liquid, transparent crystalline plates, transparent gel, and the white material which seems to be an amorphous solid dispersed throughout the gel. Our observations are shown in Table 1.

After initial preparation, the gel took 2–3 d to form. If the gel was broken by shaking or slight warming, it reformed in a matter of minutes to hours, but if warmed excessively gel formation required several days. The upper surface of the gel was always coated with a small amount of liquid, presumably due to temperature fluctuations and handling. In one case, however, (4.62% cholesterol at 4 °C) substantial amounts of the liquid and gel phases can coexist.

**Table 1** Coexisting states in the cholesterol-isopropanol system

| % Cholesterol (w/w) | –10 °C | 4 °C   | 18 °C | 22 °C |
|---------------------|--------|--------|-------|-------|
| 4.03                | L+P    | L      | L     | L     |
| 4.16                | L+P    | L      | L     | L     |
| 4.62                | L+P    | L+P+G  | L     | L     |
| 5.29                | L+P    | P+G    | L     | L     |
| 5.49                | L+P    | P+G+W* | L     | L     |
| 6.69                | W      | W      | W     | W     |
| 7.61                | W      | W      | W     | W     |
| 11.66               | W      | W      | W     | W†    |

L, Liquid; P, crystalline plates; G, transparent gel; W, white mass.

\*Small flakes of white appeared in the transparent gel after approximately one week, and seemed to be slowly increasing in size.

†This sample liquified at 43 °C.

Samples with 6.69% or more cholesterol form the white mass at –10 °C, but it is unlikely that this is an equilibrium state. There seems to be kinetic competition between formation of the white material and formation of the crystalline plates. Samples of 5.49% and 6.69% cholesterol were allowed to gel at 4 °C, both with a small amount of white 'snowflakes'; the 5.49% sample also contained some transparent plates. When these samples were moved to the –10 °C environment, the less concentrated sample formed plates with liquid, and the more concentrated sample formed the white mass, possibly containing some crystallites.

To find out more about the white materials we have observed, we have begun measurements of the weight gain of cholesterol on exposure to isopropanol vapours. We have observed a gain corresponding to 1.9 molecules of isopropanol per molecule of cholesterol above pure liquid isopropanol, and some of our measurements indicate that the ratio of 0.5 isopropanol molecules per cholesterol molecule may be a stable configuration.

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## Errata

In the letter 'Upper limits for the radio pulse emission from exploding black holes' by W. P. S. Meikle, *Nature* **269**, p. 41, in paragraph 3, line 15, for  $t_p$  read  $t_D$ ; in paragraph 3, line 17, for  $t$ , read  $t_p$ ; in paragraph 4, line 10, for  $\nu \approx 200$  MHz, read  $\nu \lesssim 200$  MHz; in paragraph 4, equation (II) for  $R_m \approx 5 \times 510^{21}$ , read  $R_m \approx 5 \times 10^{21}$ ; references 6 and 7 should read <sup>6</sup> Colgate, S. A. & Noerdlinger, P. D. *Astrophys. J.* **165**, 509–521 (1971) and <sup>7</sup> Colgate, S. A. *Astrophys. J.* **198**, 439–445 (1975).

In the letter 'Electrical stimulation of denervated muscle reduces incorporation of methionine into the ACh receptor' by C. G. Reiness & Z. W. Hall, *Nature* **276**, p. 655, the correct order of authors' name was transposed.

## Corrigenda

In the letter 'Aflatoxin B<sub>1</sub>-oxide generated by chemical or enzymic oxidation of aflatoxin B<sub>1</sub> causes guanine substitution in nucleic acids' by C. N. Martin & R. C. Garner, *Nature* **267**, p. 865, the legend to Fig. 1, line 14 should read . . . To this two-phase system was added 1.25 mg 3-chloro-perbenzoic acid (4 molar excess) and the whole stirred for 25 h at room temperature in the dark in a stoppered flask.

In the letter 'On the optical identifications of five X-ray sources' by H. V. Bradt *et al.*, *Nature*, **269**, p. 21, the last line of the abstract should read . . . suggested class of Be-star X-ray emitters.

# reviews

## Reproductive physiology in the male

B. P. Setchell

*The Process of Spermatogenesis in Animals.* By Edward C. Roosen-Runge. Pp. viii+214. (Cambridge University: Cambridge, London and New York, 1977.) £15.50.

This book is an extremely welcome addition to the literature on reproduction in the male. It brings together an absolute wealth of information on the formation of spermatozoa in lower animals and then relates this to the much greater body of knowledge about testis function in mammals. The book begins with a historical introduction, important in a subject which, has developed explosively in the last ten years. The author then works systematically through the Animal Kingdom, collating and tabulating the widely scattered information, and more importantly, discussing its relevance to the situation in other classes of animals. As a scientist working on the physiology and biochemistry of the testis in mammals, I found the book extremely interesting and stimulating.

The invertebrate species, in particular, provide so many extraordinary variants on the basic theme that I will look with new eyes at my own data to see whether things in mammals are as simple as they have seemed till now. I found the description of the 'carrier' spermatozoa in the marine snail *Janthina* particularly intriguing, as was Professor Roosen-Runge's own work on the marine hydrazoon *Phialidium*. This animal releases spermatozoa twice daily at sunrise and sundown for 3-4 months and its testes continue to produce and release spermatozoa *in vitro* although in these conditions spermatogonia are not renewed. This animal should obviously be studied in greater detail in view of the failure so far to maintain spermatogenesis, particularly the meiotic divisions, in cultures of fragments of mammalian testes.

I am not quite so sure whether a zoologist, who wanted to obtain a general picture of reproduction in male mammals to relate to the animals he was studying, would find all the information he needed. Professor Roosen-Runge is one of the most imaginative of the still-active older scientists in this field and he has worked for many years on spermatogenesis in mammals, although his contribution is often underrated. It is therefore a pity that

he feels that this field "has been reviewed almost to excess" and does not attempt a detailed exposition of his view of the present state of knowledge of mammalian spermatogenesis.

The book is very well produced, although I do find it irritating that the plates are grouped together rather than spaced through the text, but I suppose that this is a consequence of rising costs. There seem to be very few spelling mistakes (even fewer if the 'bettle' on p70 is not meant to be a 'beetle'). Some of the line drawings could be clearer and several are inadequately labelled, for example Fig. 3

(p7), in which the numbers are undefined and cannot be reconciled easily with the text.

In summary, this is a book which every library interested in the physiology of reproduction should have; and almost all workers in this field would learn something new by reading it. Professor Roosen-Runge is to be congratulated. I do not know of anyone else who could have done it as well. □

*B. P. Setchell is a Senior Principal Scientific Officer at the ARC Institute of Animal Physiology, Babraham, Cambridge, UK.*

## Human variation and adaptation

*Population Structure and Human Variation.* International Biological Programme. Vol. 11. Edited by G. A. Harrison. Pp. 342 (Cambridge University: New York, London and Cambridge, 1977.) £17.50.

THIS monograph consists of a selection of studies of human populations carried out during the International Biological Programme. Each of the 12 chapters attempts a broad review of investigations concerned with population genetics, demography and ecology as they relate to human variation and adaptation.

In the past, physical traits (such as head shape and skin colour) have been largely used for defining population groups, but the value of such traits is somewhat limited because they are polygenically determined, and at least some are significantly affected by the environment. For these reasons anthropologists and population geneticists have increasingly turned their attention in recent years to monogenically determined biochemical markers (blood groups, haemoglobins, serum proteins and enzymes) and it is with the distribution of these markers, as well as disease incidences and various physiological parameters, that this book is also concerned.

The world distribution of genetic markers is reviewed and also the findings in various "isolates" in North Asia, the Yanomama Indians of Brazil and the Solomon Islanders. The nature of specific factors which may influence

human variation and adaptation are also approached through the study of migration of genetically similar groups into different environments (Tokelau Islanders of the Pacific into New Zealand, the African savanna dwellers into the equatorial rain forests) the study of genetically different groups living in similar environments (various Jewish communities in Israel) and physiological adaptations to extreme environments (the semi-arid regions of Central Africa and the tropical forests of Papua New Guinea).

There are those who might question the merit of studying the frequencies of various traits in different populations which, at their lowest level, Mall the anatomist might somewhat irreverently have referred to as 'brick-counting-research'. But in defence of such studies it can be asked how else can human evolution and adaptation be investigated? If there is a defect perhaps it lies not so much in the collection and analysis of such data but rather in their interpretation.

This is essentially a postgraduate text, and in any event is far too expensive for possible recommendation to undergraduates. It is well edited and presented, and should prove particularly valuable for research workers. It is also an excellent sourcebook of relevant data for teachers of human biology, population genetics and anthropology.

**Alan E. H. Emery**

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## Crystal structure compendium

*Three-Dimensional Nets and Polyhedra.* By A. F. Wells. Pp. 268. (Wiley Interscience: New York and London, 1977.) £22; \$36.

WE are much indebted to A. F. Wells for his massive volumes of *Structural Inorganic Chemistry* which systematise the spatial arrangements of atoms in crystalline inorganic solids. Professor Wells has now taken the topological material involved in these structures and examined it in greater detail.

He considers primarily nets which are periodic in three dimensions. These are characterised topologically with symbols derived from those of Schläfli: the indices ( $p, q$ ) meaning that  $q$  links meet at each point and that the shortest circuits including any pair of links from a point have  $p$  edges. This symbol he finds to be inadequate for complete description, and further numbers are added, although it is not perhaps easy enough for the reader to grasp the significance of the sub- and superscripts, especially when dipping into the book. Perhaps about a hundred different nets are considered so that the subject is a considerable test of the spatial imagination. As Wells admits, the various structures which he describes are difficult to apprehend and the building of models is recommended to supplement the stereo photographs provided. The latter are not very successful because the screen pattern becomes intrusive when they are magnified by a stereo-viewer.

The most interesting section is on infinite polyhedra, the simplest of

which were discovered by Petrie and Coxeter in 1926. These can be obtained by inflating the links in a network until they become tubes. The resulting surface is then tiled with polygons. Such polyhedra divide space into two regions. Some molecular sieve crystals are of this type. It is clear that these figures are just the first of a hierarchy, and they begin to hint at the development of 'biogeometry', which may describe structures such as membranes, micelles, butter, the frameworks observed by electron microscopy in wood, moving up to cell organelles, and so on.

There is scope for the development of the notation and presentation of data on these networks and there are further topics, especially that of random networks, which we would like to hear about, but which the author has consciously eschewed.

The constraints which sheer topology imposes on spatial structure are remarkably strong, both in detail for atomic networks and statistically, as in the phase rule of physical chemistry. Wells has made a start on the systematisation of important structural invariants and it is to be hoped that this will eventually lead to their more complete characterisation, such as has been done for the symmetry groups by the International Tables for X-ray Crystallography. Until such time, Wells' compendium will be of value to those dealing with crystal structures and suggestive in many other areas concerned with three-dimensional structure.

A. L. Mackay

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## Computational fluid dynamics

*Numerical Methods in Fluid Dynamics.* By Maurice Holt. Pp 253. (Springer: Berlin and New York, 1977.)

THIS monograph is based on lectures from a course for graduate engineers. The emphasis is on methods developed in the USSR. For problems governed by hyperbolic partial differential equations, methods attributable to Godunov, the BVLR method, and methods using the theory of characteristics are described. The number of application studies is noticeably smaller in chapters dealing with hyperbolic problems than is the case for the later chapters dealing with the method of integral relations, Telenin's method, and the method of lines.

More than half of the book is devoted to applications of the method of integral relations, Telenin's method, and the method of lines to flows described by elliptic, parabolic, or mixed elliptic/hyperbolic partial differential equations. Two-dimensional incompressible flow problems, problems associated with blunt bodies at incidence, three-dimensional cone-field problems, and viscous laminar boundary layer problems are amongst those considered. The detailed explanations of the implementation of the methods and the many specimen results, should make it relatively easy for the inexperienced to use the methods described.

The careful presentation of variations on methods for the successful solving of particular problems, and the many references ensure that the monograph will be a welcome addition to the computational fluid dynamics literature. The text can readily be understood by anyone having a basic knowledge of the equations governing fluid flow. It will be useful both to graduate students in engineering or applied mathematics and to research workers.

Professor Holt's monograph is, however, not a model work. More authoritative criticisms of methods, together with results demonstrating the breakdown of methods, would have been desirable to avoid giving the reader the unfortunate impression that he has been provided with infallible recipes which can be used to solve any given problem.

F. Walkden

*F. Walkden is Director of the Fluid Mechanics Computation Centre at the University of Salford, where he is Professor of Applied Mathematics.*

## Arab survey

*Scientific Results of the Oman Flora and Fauna Survey 1975. A Journal of Oman Studies Special Report.* Introduced by David L. Harrison. Pp. 267. (Michael Rice: London; Ministry of Information and Culture: PO Box 600, Muscat, Sultanate of Oman, 1977.) \$10; Rials Omani 5.

THE change in fortune of the peoples of Arabia are the talk of the world today: less well known are the peninsula's untrampled desert plains and mountains which are among the few wilderness areas left on earth for scientists to explore. For example the Sultanate of Oman in south-eastern Arabia, with its backbone of mountains up to 3,000 m high, offers unique oppor-

tunities for naturalists to record plant and animal life in habitats which until a few years ago were largely inaccessible.

Oman is in a critical zoogeographical position between Eurasia and Africa and also offers in the cooler highlands of its northern parts an environment in which relict forms have survived from more temperate conditions. This useful survey, the first of its kind to be carried out in the country, has provided many species and forms new to science and has laid the foundations for conservation programmes in the Jabal Akhdar and Jabal Aswad ranges where the rare Arabian tahr (*Hemitragus jayakari*) still survives. Sarah Bunney

*Sarah Bunney, a zoologist, recently returned from Oman, where she spent two years working as librarian for the Omani Ministry of Health.*

## Control of eye movements

*The Theory of Binocular Vision.* By E. Hering. Edited by B. Bridgmann and Lawrence Stark. Pp. 218. (Plenum: London and New York, 1977.) \$29.40.

THIS admirable translation of Hering's monograph on the control of eye movements will be of unfaded value to present-day workers in the field; but it will also interest historians of science, for Hering prepared his monograph largely in response to Helmholtz's *Physiological Optics*, and it illustrates the nature of the systematic controversy between the two physiologists. The translation will help to dispel the two textbook myths, that Hering eschewed detailed experiments and that he adopted a rigidly nativist position; and it serves to emphasise how much Helmholtz and Hering shared—common problems, common assumptions, common experimental techniques and common criteria for the acceptability of evidence.

Central to the monograph are the

idea that the two eyes should be treated as a single organ (the *Doppel-auge*) and the principle that corresponding muscles are always equally innervated, whatever the initial position of the eyes before a movement (Hering's Law). In opposing the Helmholtzian view that the two eyes come to be used together only by habit, Hering is led to discuss the movements of occluded, suppressed and blinded eyes; asymmetrical vergence movements; cases of unilateral paresis of eye muscles; and the coordination of the eyes in infants.

B. Bridgmann's translation is both careful and readable (although some obscurities survive in chapter 17) and he has added a helpful Introduction. L. Stark has independently provided summaries and commentary interleaved in the main text. Internal evidence suggests a curiously strained relationship between the two editors and this is to be regretted, for the task was a very worthwhile one. **J. D. Mollon**

*J. D. Mollon is Lecturer in Experimental Psychology at the University of Cambridge, UK.*

## Inhomogeneous optical waveguides

*Inhomogeneous Optical Waveguides.* By M. S. Sodha and A. K. Ghatak. Pp. x+269. (Plenum: New York and London, 1977.) \$35.40.

ELECTROMAGNETIC THEORY as formulated by Maxwell has been with us for a good hundred years but the interest in it has shown no signs of abating. New potential applications (in this case the promise of optical communications) have brought the need for new analyses which after a period of digestion could be profitably presented in book form. The authors of this book are particularly suited for the task of writing on inhomogeneous optical waveguides since they have themselves written no less than twenty papers on the subject. The product is a book which anyone interested in the mathematical approaches is well advised to study. Apart from a few omissions (for example, the complex ray method) the authors provide a detailed and interesting review.

The strength of the book lies in giving copious answers to the question 'how to solve it?', its weakness is that the equally important question of 'what is it good for?' is sadly neglected. After all, the sole justification for the existence of these waveguides

is that they can perform some useful function. An engineer would like to know why certain structures have been chosen, what are their comparative advantages and disadvantages, and would like to see the conclusions presented in simple form. Unfortunately, this kind of discussion is entirely missing and 25-page appendix on manufacturing techniques is hardly a compensation for it. The descriptions are so sketchy and the links with the calculations so tenuous that they could have been replaced by a list of references.

There are also some minor inconsistencies in the presentation. For example, under the heading of "General Theoretical Considerations" in section 2.2, the particle current is taken as zero and the solution in section 2.3 is obtained on that assumption. In section 2.4, however, complex dielectric constants suddenly appear, and the reader is told without any explanation that the real part of the dielectric constant of a metal is negative. Such minor flaws are likely to confuse undergraduates and less well equipped graduate students. On the whole, however, the book may be recommended to those already familiar with a wide range of electromagnetic problems and wishing to move into this new field.

**L. Solymar**

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## Cell and tissue proteinases

*Proteinases in Mammalian Cells and Tissues.* Edited by A. J. Barrett. Pp. xx + 735. (North-Holland: New York, Oxford and Amsterdam, 1977.) Dfl. 196; \$79.95.

THE scope of this long and expensive book is somewhat wider than is indicated by its title because two of its sixteen chapters deal with proteinase inhibitors and another is devoted to synthetic substrates and inhibitors. In addition, the properties of the plasminogen activators of plasma, tissue and urine are described in an interesting chapter by Christman, Silverstein and Acs, who emphasise that neither the source of the plasminogen activators of the plasma, nor the identity of these enzymes with their tissue counterparts, can be stated with certainty.

Evidently, it is difficult to decide on all the enzymes which should be considered to be either cell or tissue proteinases. Undoubtedly, however, the decision to include consideration of certain plasma constituents has proved fruitful because, without data of this kind, discussion of the physiological roles of the cell and tissue proteinases would be difficult. The discussion of some aspects of how the tissue proteinases may be expected to function *in vivo*, such as whether plasminogen activators are important in tumorigenesis and wound healing, is speculative, but the inclusion of these concepts adds greatly to the interest of the book.

The rapidity at which information on tissue proteinases has accumulated since 1975 is mentioned in the preface. It is indeed remarkable that, whereas only six enzymes were dealt with in a similar book published in that year, details of seventy-one are to be found in the present volume. The introductory chapter by Barrett on the history and classification of the tissue proteinases helps to clarify the subject. By way of an extra, it is interesting to learn that Darwin in 1875 was the first to pose the question of the evolutionary relationship of the proteinases of plants and animals.

Perhaps because of the importance of elastase and Cathepsin G in inflammation the first chapter by Starkey is devoted to the methods of isolation and properties of these two enzymes which together have been shown to be capable of hydrolysing all the major tissue proteins. Particularly when extracellular (as, for instance, at the start of inflammation and thus not subject to the same degree of inhibition by  $\alpha_2$

macroglobulin or  $\alpha_1$  antitrypsin), these enzymes have been shown to be capable of digesting immune complexes and probably of playing a part in combating bacterial infection. These findings are mentioned mainly as an example of the mass of detailed information which has been brought together in this book. Undoubtedly the efforts of the many contributors have resulted in a volume which is both stimulating and will remain as a useful source book.

It is a pleasure to be able to report that there are few errors: in fact only one of any importance has been detected by the reviewer. This concerns the  $\alpha_1$  and  $\alpha_2$  macroglobulins of rabbit and rat. Unfortunately on p670, lines 7 and 8, the subscripts have been transposed. This is of some importance because as printed the impression is

given that  $\alpha_2$  macroglobulin of rabbit and  $\alpha_1$  macroglobulin of rat increase in concentration during inflammation. In fact, it is the other way round.

It will be an added recommendation to potential readers that more than half of the chapters have been written either by the editor himself or by other members of the staff of the Strangeways Laboratory. In an important sense, the appearance of this volume will make more easily available much of the research on tissue proteinases for which the Strangeways has become an acknowledged centre.

A. H. Gordon

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## Asymmetric reactions

*Stereo-Differentiating Reactions: The Nature of Asymmetric Reaction.* By Yoshiharu Izumi and Akira Tai. Pp. ix + 334. (Kodansha/Academic: Tokyo, New York and London, 1977.) \$29.50; £20.95.

FROM the introduction, it would seem that this book has two main aims: (1) to provide an introductory text with the basic physics and mathematics appropriate to asymmetric reactions; and (2) to present a new system of classification of asymmetric reactions developed by the authors and previously published in Japanese. One wonders what kind of reader the authors had in mind, since they have written a book which anyone unfamiliar with the field would have considerable difficulty in following and which therefore cannot be regarded as an introductory text. A research worker interested in the mechanism of asymmetric reactions, on the other hand, will hardly want a research monograph to contain a chapter "Basic Principle of Optical Activity" or even one on experimental methods.

The key chapters are those in which the authors give their new nomenclature and classification. The authors' exposition is sometimes difficult to follow. They write: "A stereoselective reaction is defined as one in which one specific stereoisomer is produced to a greater extent than the other", but give as an example, the conversion of maleic and fumaric acids to maleic anhydride! They then go on to introduce terms like enantiozeroplane, enantiotopos, enantioface, or diastereotopos-differentiating reaction, and the

reader is bound to wonder if these terms are necessary. Are they really useful? Does their use clarify problems in reaction mechanisms?

The new classification with six reaction types could have some advantages when considering mechanistic problems. The chapters which discuss these in detail, and which form the bulk of the book are, however, disappointing. They contain a mass of information about organic reactions and about mechanisms proposed in the literature. The extensive discussion of the authors' own work on modified Raney nickel catalysts provides an interesting test case, and it must be said that nothing has been written which could not have been written as clearly or indeed more clearly without recourse to their terms.

One is left with the feeling that the authors have made a brave effort to think about problems which are conceptually notoriously difficult, and that their ideas are not without value. Their own exposition, however, is unconvincing and it would have been far better to have written a much shorter book with fewer examples to try and make their concepts clear.

The occasional absence of the definite article indicates that the translator was Japanese; on the whole, the translation is good, but there are quite a lot of misprints. The index is inadequate; for example, one could not discover from it that Grignard reactions are twice discussed, the only entry being a subheading under "enantioface-differentiating reaction".

T. L. V. Ulbricht

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# announcements

## On the Move

**T. J. Martin**, Professor of Chemical Pathology, University of Sheffield, to Professor of Medicine, University of Melbourne, Repatriation General Hospital, Australia, from 1 October 1977.

**N. H. Hunt**, Department of Chemical Pathology, University of Sheffield, to Department of Experimental Pathology, John Curtin School of Medical Research, A.N.U., Australia.

Details of changes of department, sabbaticals, where leave will be taken and so on should be sent to On the Move; there is no charge for this service.

## Appointments

**Dr D. F. Hornig**, President-emeritus of Brown University to Professor of Chemistry in Harvard Faculty of Public Health to direct development of a proposed University-wide undertaking, Interdisciplinary Programs in Health.

**Mr L. R. Hanton**, University of Otago, New Zealand, to a Rutherford Scholarship for three years from 1 October 1977 to work in the department of organic and inorganic chemistry, University of Cambridge.

## Awards

**Dr D. Schofield**, chief of the Defence Research Establishment at Dartmouth, N.S. to deputy chief of laboratories, responsible for all Department of National Defence research establishments. Dr Schofield will be replaced by F. A. Fergusson, Deputy Chief in Ottawa.

**Dr S. Weinberg**, Higgins Professor of Physics at Harvard University and Senior Scientist at the Smithsonian Astrophysical Observatory, is the 1977 winner of the American Institute of Physics-United States Steel Foundation Science-Writing Award (\$1,500) for his book, 'The First Three Minutes', relating the theory of elementary particles to the problem of the very early moments of the Universe.

## The British Council

### East European Exchange Visits

Cultural agreements with the Soviet Union, Czechoslovakia, Hungary, Romania and Bulgaria provide for short visits (usually 2 weeks) and medium term research visits (2-6 months) for British scientists. Scholarships are also available for periods of up to one academic year. For full

details and application form, contact Higher Education and Science Department, The British Council, 10 Spring Gardens, London SW1, UK.

## Meetings

21-22 October, **International Symposium on Chromogenic Substrates**, London (Thrombosis Research Unit, Department of Surgery, Denmark Hill, London SE5, UK).

2-4 November, **Symposium on Advanced Ozone Technology**, Toronto (A. Netzer, Head, Physical Chemical Processes, Wastewater Technology Center, Canadian Center for Inland Water, PO Box 5050, Burlington, Ontario, Canada).

2-5 November, **Symposia on Perspectives in Biochemistry, and Isoenzymes**, Cordoba, Argentina (R. Caputto, Departamento Quimica Biologica, Facultad de Ciencias Quimicas, Universidad Nacional de Cordoba, Ciudad Universitaria, 5000-Cordoba, Argentina).

9 November, **Liquid Crystals**, London, UK (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

9 November, **Symposium on Humidity Measurement**, London, UK (Sira Institute Ltd, South Hill, Chislehurst, Kent, UK).

## Person to Person

Mathematician seeks family accommodation in Oxford, January to December 1978, or exchange house in Newcastle, N.S.W., Australia. Contact W. Brisley, Mathematics Department, University of Newcastle, N.S.W. 2308, Australia.

There will be no charge for this service. Send items (not more than 60 words) to Marcus Dobbs at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

10-12 November, **Open Conference on the Implications of Recombinant DNA Research**, Bloomington, Indiana (Professor R. P. Bareikis, The Poynter Center 410 North Park Street, Bloomington, Indiana 47401).

14-15 November, **New Developments in Immunoassays**, Düsseldorf (M. A. Andreopoulos, Conference Director, Robert S. First, Inc., Avenue Marnix 19A, Box 6, 1050 Brussels, Belgium) (also in Chicago, 5-6 December).

14-15 November, **Symposium on Mass Spectrometry and Combined Techniques in Medicine, Clinical Chemistry**

**and Clinical Biochemistry**, Tübingen (Medizinische Klinik, Universität Tübingen, Otfried-Müller-Strabe 10, 7400 Tübingen, GFR).

14-17 November, **International Symposium on the Pineal Organ**, Jerusalem (I. Nir, Faculty of Medicine, Hadasseh Medical School, Jerusalem, Israel).

14-18 November, **The Pan American Conference on Forensic Applications of Anthropology, Dentistry, Medicine and Palaeopathology**, Mexico City (W. G. Eckert, Laboratory, St Francis Hospital, Wichita, Ks. 67214).

17-18 November, **Symposium on Nutritional Management of Genetic Disorders**, New York (The National Foundation-March of Dimes, 622 Third Avenue, New York, N.Y. 10017).

21-26 November, **Fifth International Conference on Global Impacts of Applied Microbiology**, Bangkok (Chairman, GIAM V, National Organising Committee, Mahidol University, GPO Box 4-130, Bangkok, Thailand).

22-23 November, **Symposium on Photography in the Laboratory**, London (The Secretary, Scientific Symposia Ltd, 42-43 Gerrard Street, London W1, UK).

## Reports and Publications Other Countries—July

United States Department of the Interior: Geological Survey. Professional Paper 655-M: Effects of Phreatophyte Removal on Water Quality in the Gila River Phreatophyte Project Area, Graham County, Arizona. By R. L. Laney. Pp. iv+23+2 plates. (Washington, DC: US Government Printing Office, 1977.) [117]

CERN — European Organization for Nuclear Research. CERN 77-10: Selection of Formulae Concerning Proton Storage Rings. By G. Guignard. Pp. x+108. (Geneva: CERN, 1977.) [117]

Deutsches Hydrographisches Institut, Hamburg. 29./30. Jahresbericht 1974/1975. Pp. 134. (Hamburg: Deutsches Hydrographisches Institut, 1977.) [127]

Bayer 1976. Pp. 103. (Leverkusen: Bayer Aktiengesellschaft, 1977.) [127]

National Multiple Sclerosis Society. 1976 Annual Report. Pp. 32. (New York: National Multiple Sclerosis Society, 205 East 42nd Street, 1977.) [137]

CERN — European Organization for Nuclear Research. CERN 77-11: Future Photomultiplier Assemblies and Associated Electronics in Large Experiments. By P. Duteil, R. Hammarström, P. G. Innocenti, A. Michelini, B. Smith and F. Soso. Pp. vi+35. (Geneva: CERN, 1977.) [147]

United States Department of the Interior: Geological Survey. Bulletin 1422-B: The Maudlow and Sedan Formations of the Upper Cretaceous Livingston Group on the West Edge of the Crazy Mountains Basin, Montana. By Betty Skipp and L. W. McGrew. Pp. iv+68. Professional Paper 608-B: General Geology and Petrology of the Precambrian Crystalline Rocks, Park and Jefferson Counties, Colorado. By C. C. Hawley and R. A. Wobus. Pp. v+77+plate 1. Professional Paper 984: Geology of an Upper Cretaceous Copper Deposit in the Andean Province, Lassiter Coast, Antarctic Peninsula. By Peter D. Rowley, Paul L. Williams and Dwight L. Schmidt. Pp. vi+36. (Washington, DC: US Government Printing Office, 1977.) [147]

Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem. Heft 174: Gaschromatographie der Pflanzenschutzmittel—Tabelle der Literaturreferate VI; Kumulative Indices 1-VI. Von Dr. Winfried Ebing. Pp. 102. (Berlin-Dahlem: Biologischen Bundesanstalt für Land- und Forstwirtschaft, 1977.) DM 17.50. [187]

Journal of Mechanical Working Technology, Vol. 1, No. 1, July 1977. Published Quarterly. Pp. 1-114. Annual Subscription: Dfl. 155; \$58.95. (Amsterdam: Elsevier Scientific Publishing Company, 1977.) [187]

## UK &amp; Ireland—August

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- The Electricity Council Annual Report, 1976-1977. Pp. 24. (London: The Electricity Council, 30 Millbank, SW1, 1977.) [18]
- Chinese Astronomy*, Vol. 1, No. 1, June 1977. (A selected translation of *Acta Astronomica Sinica*, Vols. 15-17, 1974-1976.) Pp. 1-196. Annual subscription for 1977: \$105. (Oxford and New York: Pergamon Press, 1977.) [18]
- Writing a Scientific Paper. By Vernon Booth. Fourth edition. Pp. 32. (London: The Biochemical Society, 1977. Published by permission of Koch-Light Laboratories, Ltd.). £1.52. [38]
- Office of Population Censuses and Surveys. Mortality Statistics: Cause. (Review of the Registrar General on Deaths by Cause, Sex and Age, in England and Wales, 1975. Series DH2, No. 2.) Pp. vi + 82. (London: HMSO, 1977.) £2 net. [38]
- The Determination of Vinyl Chloride: a Plant Manual. (Analytical Methods Required for the Control of Vinyl Chloride Concentrations in and Around Manufacturing and Process Plants.) Compiled by specialists convened by the Vinyl Chloride Committee of the Chemical Industries Association, Ltd., and edited by W. Thain. Third edition. Pp. 154. (London: Chemical Industries Association, Ltd., 93 Albert Embankment, SE1, 1977.) CIA members £15; non-CIA members £20. [48]
- The Malaysian Rubber Producers' Research Association. Thirty-ninth Annual Report, June 1977. Pp. 28. (Brickendonbury, Hertford: The Malaysian Rubber Producers' Association, 1977.) [48]
- The Natural Environment Research Council. Post-graduate Training in the Sciences of the Natural Environment. Pp. i + 9. Research Grants in the Natural Environmental Sciences—NECR Research Grants Current on 1st October 1976. Pp. v + 60. (London: The Natural Environment Research Council, 1977.) [48]
- Department of Industry. Research and Development Requirements Boards, Reports 1976-1977. Pp. 40. (London: Department of Industry, 1977.) [48]
- International Planned Parenthood Federation. Occasional Essay No. 4: Defining Family Health Needs, Standards of Care and Priorities, with particular reference to Family Planning. By Fred T. Sai. Pp. 32. (London: International Planned Parenthood Federation, 18-20 Lower Regent Street, SW1, 1977.) 85p; £1.45. [48]
- Gesellschaft für Strahlen- und Umweltforschung, München. Jahresbericht 1976. Pp. 312. (München: Gesellschaft für Strahlen- und Umweltforschung, 1977.) [187]
- National Research Council Canada. Report of the President, 1976/1977. Pp. 108. (Ottawa: National Research Council Canada, 1977.) [187]
- Smithsonian Contributions to Zoology. No. 244: A Review of the Troglobitic Decapod Crustaceans of the Americas. By Horton H. Hobbs, Jr., H. H. Hobbs, III, and Margaret A. Daniel. Pp. v + 183. No. 248: Redescription of *Echinoderes dujardini* (Kinorhyncha) with Descriptions of Closely Related Species. By Robert P. Higgins. Pp. iii + 26. (Washington, DC: Smithsonian Institution Press, 1977. For sale by US Government Printing Office.) [207]
- World Health Organization. Technical Report Series. No. 608: Criteria for the Evaluation of Learning Objectives in the Education of Health Personnel—Report of a WHO Study Group. Pp. 47. (Geneva: WHO; London: HMSO, 1977.) Sw.fr. 6; \$2.40. [217]
- Economic and Social Commission for Asia and the Pacific. Committee for Co-ordination of Joint Prospecting for Mineral Resources in Asian Offshore Areas (CCOP). Technical Bulletin, Vol. 10. Pp. v + 212. (Tokyo: Sumitomo Printing and Publishing Co., Ltd., 3-2 Kanda Jinbo-cho, Chiyoda-ku, 1976.) [217]
- World Health Organization. Technical Report Series. No. 605: Chemotherapy of Solid Tumours—Report of a WHO Expert Committee. 106. (Geneva: WHO; London: HMSO, 1977.) Sw.fr. 10; \$4. [257]
- Massachusetts Institute of Technology: Lincoln Laboratory. Technical Note 1977-24: Station Magnitude Bias—Its Determination, Causes, and Effects. By R. G. North. Pp. v + 62. (Lexington, Mass.: Lincoln Laboratory, MIT, 1977.) [257]
- World Health Organization. Technical Report Series. No. 607: WHO Expert Committee on Leprosy—Fifth Report. Pp. 48. (Geneva: WHO; London: HMSO, 1977.) Sw.fr. 6; \$2.40. [267]
- Unesco. Intergovernmental Oceanographic Commission. Technical Series No. 12: Oceanographic Products and Methods of Analysis and Prediction. Pp. 172. (Paris: Oecd, 1977.) [267]
- United States Department of the Interior: Geological Survey. Professional Paper 1014: Late Quaternary Depositional History, Holocene Sea-Level Changes, and Vertical Crustal Movement, South San Francisco Bay, California. By Brian F. Atwater, Charles W. Hedel and Edward J. Helley. Pp. v + 15 + plate 1. (Washington, DC: US Government Printing Office, 1977.) [267]
- Annual Report of the Inter-American Tropical Tuna Commission, 1976. Pp. 180. (La Jolla, California: Inter-American Tropical Tuna Commission, c/o Scripps Institution of Oceanography, 1977.) \$2. [277]
- Lawrence Livermore Laboratory. First Annual Report to the High Altitude Pollution Program. Frederick M. Luther: Principal Investigator. Pp. 48. (Washington, DC: US Department of Transportation, Office of Environmental Quality, 1977.) [277]
- Australia: Commonwealth Scientific and Industrial Research Organization. Division of Soils Technical Paper No. 32: Some Aspects of Genesis of a Red Earth Profile at Townsville, Queensland. By R. Brewer and R. J. Coventry. Pp. 21. (Melbourne: CSIRO, 1977.) [287]
- Government of India. Ministry of Health and Family Planning. Report of the Central Drugs Laboratory, 1st April 1971 to 31st March 1974. Pp. i + 60. (Calcutta: Central Drugs Laboratory, 1976.) Rs. 14; £1.64; \$5.04. [287]
- Israel. The National Council for Research and Development Advances in Cloud Physics and Precipitation Stimulation in Israel. (2nd Israeli Symposium.) Pp. 97. Advances in Israel Research on Water Desalination. (Lectures given by the Israeli Scientists at the International Desalting and Environmental Association Conference, held in Mexico City, Mexico, 24-19 October, 1976.) Pp. 73. (Jerusalem: National Council for Research and Development, 1977.) [287]
- Lautäusserungen und Lauterkennen bei Insekten (Grillen). Von Franz Huber. (Rheinisch-Westfälische Akademie der Wissenschaften: Natur-, Ingenieur- und Wirtschaftswissenschaften, Vorträge, N. 265.) Pp. 66. (Wiesbaden: Westdeutscher Verlag, GmbH., 1977.) DM 19.80. [297]
- Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 280, No. 970: The Formation of Muscle Scars in Articulate Brachiopods. By D. I. Mackinnon. Pp. 1-12 + plates 1-11. UK £4.25; Overseas £4.40. Vol. 280, No. 971: Cranial Morphology of the Loxommatidae (Amphibia: Labyrinthodontia). By Eileen H. Beaumont. Pp. 29-101 + plates 1 and 2. UK £5.50; Overseas £5.70. (London: The Royal Society, 1977.) [48]
- The Radiochemical Centre Limited, Amersham. Sixth Annual Report, for the year ended 31st March 1977. Pp. 18. (Amersham: The Radiochemical Centre Limited, 1977.) [48]
- Philip Harris. Ltd. Science Education Equipment and Materials—Supplement and Price List, 1977. Pp. 60. (Shenstone, Staffordshire: Philip Harris, Ltd., 1977.) [58]
- National Health Service Act 1966. Report of the General Practice Finance Corporation for the period 1st April, 1976 to 31st March, 1977. Pp. 18. (London: HMSO, 1977.) 35p net. [88]
- Rowett Research Institute. Annual Report of Studies in Animal Nutrition and Allied Sciences, Vol. 32, 1976. Pp. 125. (Bucksburn, Aberdeen: Rowett Research Institute, 1977.) £1.50. [88]
- Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 287, No. 1337: The Six Roll Mill: Unfolding an Unstable Persistently Extensional Flow. By M. V. Berry and M. R. Mackley. Pp. 1-16 + plates 1-5. (London: The Royal Society, 1977.) UK £1.85; Overseas £1.95. (London: The Royal Society, 1977.) [98]
- Town and Country Planning Act 1971. Control of Office Development. Annual Report by the Secretary of State for the Environment and the Secretary of State for Wales for the year ended 31 March 1977. Pp. 8. (London: HMSO, 1977.) 25p net. [118]
- Second Report from the Select Committee on Science and Technology. Session 1976-77. A Case Study of a Machine Tool Development at the Cranfield Institute of Technology. Pp. xxiv + 132. (London: HMSO, 1977.) £3.60 net. [118]
- Computers and Chemical Engineering. Vol. 1, No. 1, 1977. Edited by Richard R. Hughes. Published quarterly. Pp. 1-100. Annual subscription: \$64. Specially reduced rates to individuals whose institution takes out a library subscription: \$30. (Oxford and New York: Pergamon Press, 1977.) [118]
- Department of Energy. Freight Transport: Short and Medium Term Considerations. (Energy Paper No. 24.) (Advisory Council on Energy Conservation.) Pp. iv + 22. (London: HMSO, 1977.) £1.50. [158]
- National Radiological Protection Board. NRPB-R62: Insignificant Levels of Dose—a Practical Suggestion for Decision Making. By G. A. M. Webb and A. S. McLean. Pp. 16. (Harwell, Didcot, Oxon: National Radiological Protection Board, 1977. Obtainable from HMSO, London.) £1. [158]
- National Radiological Protection Board. Protection Against Ultraviolet Radiation in the Workplace. Pp. 16. (Harwell, Didcot, Oxon: National Radiological Protection Board, 1977. Obtainable from HMSO, London.) 40p. [178]
- Council for National Academic Awards. Directory of First Degree Courses. Pp. 175. Directory of Postgraduate Courses, 1977-78. Pp. 84. (London: Council for National Academic Awards, 344/354 Gray's Inn Road, WC1, 1977.) [178]
- Annals of the ICRP. Vol. 1, No. 1: Radiation Protection in Uranium and Other Mines. (ICRP Publication No. 24) Pp. 28. (Oxford: Pergamon Press, Ltd., 1977. Published for the International Commission on Radiological Protection.) £3.25. [178]
- Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 280, No. 972: The Changing Environmental Conditions in Great Britain and Ireland During the Devensian (Last) Cold Stage. A Discussion organised jointly for the Royal Society and the Royal Irish Academy by G. F. Mitchell, FRS, and R. G. West, FRS. Pp. 103-374 + 1 plate. (London: The Royal Society, 1977.) UK £18.75; Overseas £19.25. [178]
- Meteorological Office. Annual Report for 1976. (Met.0907) Pp. xiv + 145. (London: HMSO, 1977.) £2.75 net. [188]
- Proceedings of the Royal Irish Academy. Section A: Mathematical and Physical Sciences. Vol. 77, No. 3: Constructing Smooth Measures on Certain Classes of Paved Sets. By P. McGill. Pp. 31-34. 98p. Section B: Biological, Geological and Chemical Science. Vol. 77, No. 4: Breccia-Pipes Associated with the Ardara Pluton, County Donegal. By W. J. French. Pp. 101-117. 84p. Vol. 77, No. 5: Contributions to the Lichen Flora of South-East Ireland—II. By M. R. D. Seaward. Pp. 119-134. 78p. Vol. 77, No. 6: The Genetics of Hatching in the Potato Cyst-Nematode *Heterodera rostochiensis* Woll. By H. El-Shatoury. Pp. 135-141. 47p. Vol. 77, No. 7: Palaeontological Evidence for the Age of the Lower Palaeozoic Rocks of the Slievean Inlier, County Tipperary. By J. R. J. Colthurst and D. G. Smith. Pp. 143-158 + 2 plates. 96p. (Dublin: Royal Irish Academy, 1977.) [198]
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- Potato Marketing Board. Maincrop Potato Production in Great Britain, 1975-6. (An Investigation by the Potato Marketing Board into the Farm Types, Production Techniques and Materials Used in Growing Maincrop Potatoes in Great Britain.) Pp. 68. (Covley, Oxford: Potato Marketing Board, 4 Between Towns Road, 1977.) 75p. [228]
- Monitoring and Assessment Research Centre of the Scientific Committee on Problems of the Environment, International Council of Scientific Unions. MARC Report No. 4: The Utility of the Nigerian Peasant Farmer's Knowledge in the Monitoring of Agricultural Resources. By David Barker, Julius Oguntuyinbo and Paul Richards. (A General Report.) Pp. 53. £1; \$2. MARC Report No. 5: Monitoring Tropical Forests—a Review with Special Reference to Africa. By Timothy J. Synnott. (A Technical Report.) Pp. 45. £1; \$2. (London: MARC, Chelsea College, 1977.) [228]
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- The Lister Institute of Preventive Medicine. Report of the Governing Body, 1977. Pp. 12. (Elstree, Herts: Lister Institute of Preventive Medicine, University of London, 1977.) [228]
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- In Defence of the NHS. Pp. 40. (London: Radical Statistics Health Group, c/o BSSRS, 9 Poland Street, London, W1, 1977.) 50p + 15p p&p. [258]
- Factors which Influence the Success of Supervisory Training. By Don Bates and Dian Hosking. (Occasional Paper No. 5.) Pp. 64. (Watford: Engineering Industry Training Board, 54 Clarendon Road, 1977.) £1.50. [228]
- Progress in Crystal Growth and Characterization*, Vol. 1, No. 1, 1977. Pp. 1-91. Published as one volume of four parts per year. Editor-in-Chief: Dr Brian R. Pamplin. 1977 Subscription rate: \$45 (including postage and insurance). Two year subscription (1977/78): \$85.50. (Oxford and New York: Pergamon Press, 1977.) [238]
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